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POSSIBLE ROLE OF PECTIC ENZYMES IN ABSCISSION[1]

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Abscission has been of interest to botanists since Mohl described it as early as 1860 (20). Many of the early workers assumed that enzymes are involved in the dissolution of the middle lamella as abscission occurs (11, 12, 16, 17). As Addicott and Lynch indicate in their review of the physiology of abscission, however, little has been done with the identification of specific enzymes which may be involved in the dissolution reactions (2). Much of the current research concerned with abscission deals with chemical substances which affect the process with little attention to specific chemical reactions that may be involved.

Osborne has shown recently that the activity of pectin methylesterase (PME) decreases when the time for abscission of leaves in *Phaseolus vulgaris* occurs normally or when abscission is induced by chemical factors (23). Since the activity of PME is affected by growth substances (4, 21, 26) which also affect abscission (1, 25, 27), a role for PME in the reactions involved in abscission is suggested.

The middle lamella is generally considered to be pectic in nature (6, 13, 18). The middle lamella of the abscission zone dissolves as floral abscission occurs in Nicotiana (12, 28). In 1950 Facey reported the results of experiments with Fraxinus cuttings which identified water soluble pectin as the end product of a series of reactions which occur as the calcium pectate of the middle lamella is dissolved during abscission (10). As early as 1918 Kendall reported the appearance of pectin as abscission occurred in tobacco flowers (12). The problem arises as to whether the shifts from pectate to pectin as the middle lamella dissolves is simply caused by a decrease in PME activity or whether additional pectic enzymes are involved.

The literature connected with pectic enzymes other than PME is conflicting, including their actual occurrence in higher plants. Pectin-polygalacturonase is the best known of these other enzymes. Kertesz is doubtful of its occurrence in higher plants although he cites several reports of its occurrence (15). Demain and Phaff recently reported that instead of a single pectin-polygalacturonase, there are several exo-polygalacturonases specific for pectins of different degrees of esterification and size of polymer and a single endo-galacturonase (7) (8). Schubert (24), Dingle et al (9), and Ayres et al (3) have reported that endo-polygalacturonase is a complex of enzymes. This complexity in the identification of specific pectin-polygalacturonases may explain some of the conflicting reports. McClendon and Somers have reported the occurrence of two pectin-glycosidases with different conditions required for activity (19). Kertesz has postulated the occurrence of additional pectic enzymes in general and describes one that he has named pectic acid depolymerase (15). Although there is general lack of information concerning pectic enzymes, it is probable that there are one or more enzymes present in abscission tissue which could act to dissolve the cementing middle lamella.

The purpose of this investigation was to examine the occurrence of PME in the abscission zone of tobacco flowers and to note effects that various substances known to affect abscission might have upon its activity (22, 29, 30). In addition, attempts were made to duplicate in vitro the macerating effects presumably caused by pectic enzymes as abscission occurs normally. These same substances which affect abscission were studied as to their effect upon the macerating activity of certain pectic enzymes.

METHODS

PME ANALYSIS Little Turkish and Lizard's Tail varieties of *Nicotiana tabacum* were used throughout the investigation in order that any varietal differences associated with self and cross pollination mechanisms could be observed. To determine the activity of PME, a cross section of tissue 1 mm thick was removed from the abscission zone of approximately 60 pedicels which abscised at touch. The PME activity in the abscission tissue was compared to the activity in tissue from the leaf, stem, petiole, and upper pedicel. The fresh weights of these tissues were recorded after which they were ground in a mortar with acid-washed white quartz sand. This preparation was added to a 1 % pectin solution containing 0.1 *N* NaCl. This mixture was agitated for 30 min while 0.1 *N* NaOH was added to maintain a constant pH 6.8 as measured by a glass electrode.

[1] Received March 16, 1959.

The relative activity was computed using the method proposed by Kertesz (14). The number of milligrams of methoxyl groups that are split off during the 30 min reaction period is obtained by multiplying the volume of alkali required by 3.1, the equivalent molecular weight of the methoxyl group. The resulting unit of relative activity is termed the PME unit per gram of fresh tissue. Effects of indoleacetic acid (IAA) and methionine upon activity of tissue from the abscission zone were determined.

Separation Activity Thin free-hand longitudinal sections measuring 5 mm in length were removed from several pedicels so that the cells of the abscission region could be observed with a microscope and compared to surrounding cells. These sections were added to solutions of various pectic enzymes. Thirty sections were incubated with 10 ml of enzyme preparation in a Petri culture at 25° C. Several sections of tissue were removed from the cultures and mounted for microscopic examination periodically. Following such examination the tissue was discarded. Visible separation of the cells was noted and the time for complete separation was compared to the time required when a different enzyme concentration, buffer, or pH level was used and when IAA and methionie were added to the media. Each experiment was duplicated ten times.

PME and a crude preparation called pectinase are available commercially and were secured from the Nutritional Biochemicals Corporation. McClendon and Somers of the University of Delaware have generously provided the two pectin-glycosidases which they secured from Rome Beauty apples that were rotted by *Botryosphaeria ribis*. The author refers to these two pectin-glycosidases as enzymes A and B. [Enzyme A is a concentrate from the pressed juice (pH 3.5) while enzyme B is a concentrate of an extract from the pressed cake after removal of the juice (pH 4.5). Enzyme A has a pectin-polygalacturonase activity (measured viscometrically with pectate pH 4) about two times that of enzyme B while the maceration activity upon tissues from tomato leaves and potato slices is ten times more active in B than A (expressed on a dry weight basis). These preparations can hydrolyze polygalacturonic acid, araban, galactan, galactomannan, xylan, and pectin at varying rates. The pectinase preparation (from NBC) is much more potent in all of these activities.][2]

Table I

Pectin-methylesterase Activity of Various Tissue Macerates

Tissue from	Activity/g fresh tissue	
	Lizard's tail	Little turkish
Abscission Zone	12.12	13.95
Upper Pedicel	10.72	11.78
Petiole	7.00	6.27
Stem	7.01	6.87
Leaf*	9.83	9.64

* Gel formation often interfered with the reaction before the 30 minute time interval was complete.

Results

PME Analysis The PME activity in the tissue of the abscission zone of the pedicel is greater than in any other tissue tested with the possible exception of leaf tissue (See table I). In this instance gel formation interfered with agitation during the 30 min interval in which 0.1 *N* sodium hydroxide was added. When IAA is added, the activity rates are altered as tabulated in table II. Differences between the effects caused by adding IAA are statistically significant as indicated in table III. Duplicate tests each with half the ground tissue produced nearly identical values for enzyme activity.

Adding 5 ppm IAA decreases PME activity. Adding 100 and 1,000 ppm IAA increases PME activity. Tests with still higher concentrations of IAA produce only slight increases in PME activity over that observed when 1,000 ppm IAA is added.

Methionine decreases PME activity when added to the culture media (see table II). This difference in PME activity between the control and the addition of 0.01 M methionine is significant at the 1 % level (see table III). Adding 1,000 ppm IAA in addition to the methionine, however, causes the disappearance of the decreased PME activity that was observed

[2] Information received through personal communication with Dr. John H. McClendon, University of Delaware.

Table II

Effect of IAA and Methionine upon Activity of PME of Macerates of Tissue from Abscission Zone

Addition to tissue	Number of trials	Mean activity/g fresh tissue (± 1 standard error)	
		Lizard's Tail	Little Turkish
0	15	12.21 ± 0.33	13.16 ± 0.12
5 ppm IAA	5	11.61 ± 0.95	12.92 ± 0.54
100 ppm IAA	5	13.20 ± 0.59	14.55 ± 0.77
1,000 ppm IAA	10	16.71 ± 0.27	16.97 ± 0.38
0.01 M Methionine	5	9.54 ± 1.00	10.65 ± 1.13
1,000 ppm IAA+ 0.01 M Methionine	5	15.32 ± 0.63	15.31 ± 0.68

TABLE III

DETERMINATION OF SIGNIFICANCE BETWEEN EFFECTS OF IAA AND METHIONINE UPON ACTIVITY RATE OF PME FROM TISSUE OF ABSCISSION ZONE

DIFFERENCES BETWEEN ADDITIONS OF	[t] VALUES	
	LIZARD'S TAIL	LITTLE TURKISH
1. 0 and 5 ppm IAA	0.67	0.60
2. 0 and 100 ppm IAA	1.33	2.67*
3. 0 and 1,000 ppm IAA	8.33***	11.20***
4. 0 and 0.01 M Methionine	2.93**	3.53**
5. 0 and 1,000 ppm IAA + 0.01 M methionine	3.74**	4.88***
6. 5 ppm IAA and 100 ppm IAA	1.28	1.27
7. 100 ppm IAA and 1,000 ppm IAA	5.83***	2.91**
8. 1,000 ppm IAA and 0.01 M methionine	8.74***	6.22***
9. 1,000 ppm IAA + 0.01 M methionine and 0.01 M methionine	6.21***	2.74*
10. 1,000 ppm IAA and 1,000 ppm IAA + 0.01 M methionine	2.28*	2.21*

* Significant at the 5 % level
** Significant at the 1 % level
*** Significant at the 0.1 % level

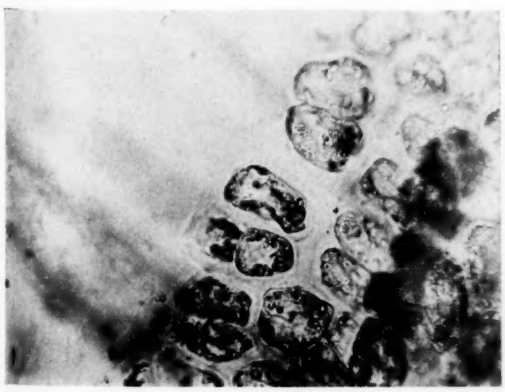
when methionine was added singly. Nearly the same increase in PME activity caused by the 1,000 ppm IAA alone is observed. Additions of 5 ppm IAA and the methionine produced no significant difference in PME activity than when the methionine was present alone.

ESTERIFICATION REACTIONS Other experiments were performed in the same manner except that 0.1 *N* hydrochloric acid was added to maintain a constant ph 4.5 during the 30 min reaction time. This presumably would be a direct test for the esterification reaction which would oppose the activity of PME.

The activity of such an esterification enzyme was confined to the first seven or eight minutes of the usual 30 min reaction time. Since the time of reaction was only one-fourth that in the other experiments, activity values were considerably smaller and there was less overall variation in activity when IAA and methionine were added. Averages of several such experiments produced figures which indicated that addition of methionine increased esterification. Additions of 1,000 ppm IAA completely inhibited esterification in Little Turkish and nearly so in Lizard's Tail tissue. Variations were slight in all of the experiments, however.

SEPARATION ACTIVITY Pectinase and enzyme B cause dissolution of the middle lamella and separation of cells of the abscission zone as well as other cells of the tissue slice through the pedicel. The dissolution of the middle lamella with these enzymes resembles the dissolution which normally occurs during abscission (28). The chief difference in this induced dissolution from that which normally occurs is the normal restriction of activity to certain tiers of cells. Since many tiers of cells are involved in the actual separation of cells in Lizard's Tail variety as compared to relatively few in Little Turkish, the pattern of separation of cells caused by pectinase and enzyme B is more like that which occurs in Lizard's Tail. The results of the incubation of tissue slices with the four pectic enzymes are tabulated in table IV. Where separation occurred (and hence dissolution of the middle lamella), no difference could be observed between the action of pectinase and enzyme B.

The action of the enzymes could be observed with a microscope. Cells near the periphery were acted upon first. Incubation was continued until the cells



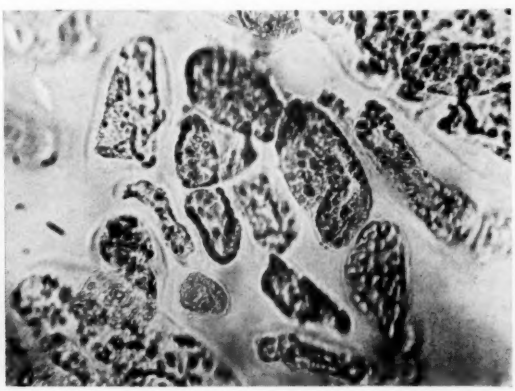


FIG. 1 (*left*). Separation of cells of the pedicel of Lizard's Tail tobacco as a result of treatment with pectinase. (x 820)

FIG. 2 (*right*). Separation of cells of the pedicel of Little Turkish tobacco as a result of treatment with pectinase. (x 820)

were separated by the weight of a cover slip when the section was mounted for microscopic examination. Figures 1 and 2 represent samples of separation of cells which were termed complete separation. The values recorded in tables IV to VII indicate the amount of time required for the enzymes to cause such separation. In several duplicated experiments variation in time did not exceed 1 hr except in a few cultures where fungal contamination occurred.

The kind of buffer and the pH affect the separation activity of pectinase and enzyme B (see tables IV and V). Separation occurs more rapidly at pH 3.5. Resulting discoloration of tissue slices, however, indicates adverse physiological effects upon the cells. No such effects are observed at pH 4.5. Higher pH levels result in sharp decreases of activity. No separation is observed at pH 6.2. The maleate buffer increases the activity of enzyme B slightly. The citrate buffer increases the activity of pectinase slightly at pH 4.5 while enzyme B is affected considerably at pH 3.5 (see table V).

Low concentrations of IAA (5 ppm) and methionine increase the activity of pectinase and enzyme B considerably (see table VI). When 5 ppm and 0.01 M methionine are added to the substrate in combination, only a slight increased activity is observed by pectinase and none by enzyme B (see table VII). By adding 1,000 ppm IAA to the substrate the action of pectinase is inhibited completely during the time limit of 56 hrs for these experiments. This inhibitory effect of 1,000 ppm IAA is still apparent when 0.01 M methionine is added as well. The effects of methionine upon rate of separation is proportional to the concentration added (see table VI).

Discussion

There is evidence that PME acts to retard or to prevent abscission. Methionine and low concentrations of IAA accelerate abscission; high concentrations of IAA retard or prevent abscission (30). Methionine and low concentrations of IAA decrease PME activity; high concentrations of IAA increase PME activity. In addition, if pectin is the end product of the abscission reactions as reported earlier (10, 12), an enzyme which tends to de-esterify pectin would act to prevent abscission. Osborne's data which show a decrease in PME activity as abscission approaches in the leaves of *Phaseolus vulgaris* is added evidence that PME acts to retard or prevent abscission. Since the action of PME is irreversible (15), it cannot act under certain conditions to produce pectin and hence cause abscission.

The existence of other pectic enzymes active in abscission seems likely. A transmethylase to provide methyl groups for the shift from pectic acid to pectin is a probable enzyme. The action of one or more pectin-polygalacturonase would certainly increase the

Table IV

Effect of Various Pectic Enzymes upon Separation of Cells of Tissue Slices from Abscission Zone

Enzyme (0.025 %)	Hrs required for separation*		
	pH 3.5**	pH 4.5	pH 6.2
No Enzyme	No separation	No separation	No separation
Pectinase (from NBC)	6	20	No separation
Pectin-methylesterase (from NBC)	No separation	No separation	No separation
Enzyme A*** (from McClendon and Somers)	No separation	No separation	No separation
Enzyme B*** (from McClendon and Somers)	56	No separation	No separation
Enzyme (0.1 %)			
No enzyme	No separation	No separation	No separation
Pectinase (from NBC)	4	8	No separation
Pectin-methylesterase (from NBC)	No separation	No separation	No separation
Enzyme A*** (from McClendon and Somers)	56	No separation	No separation
Enzyme B*** (from McClendon and Somers)	18	24	No separation

* Experiments observed for 56 hrs
** Tissue discolored in 6 to 8 hrs
*** 0.05 M Citrate buffer used

TABLE V

EFFECT OF BUFFER UPON SEPARATION ACTIVITY OF VARIOUS PECTIC ENZYME PREPARATIONS

ENZYME	pH	ENZYME CONC	HRS REQUIRED FOR SEPARATION*		
			BUFFER		
			NONE	MALEATE	CITRATE
No Enzyme	4.5	0.00 %	No separation	No separation	No separation
Pectinase	4.5	0.025 %	20	20	16
Pectin-methylesterase	4.5	0.025 %	No separation	No separation	No separation
Enzyme A	4.5	0.025 %	No separation	No separation	No separation
Enzyme B	4.5	0.025 %	No separation	No separation	No separation
No enzyme	3.5	0.00 %	No separation	No separation	No separation
Pectinase	3.5	0.1 %	4	4	4
Pectin-methylesterase	3.5	0.1 %	No separation	No separation	No separation
Enzyme A	3.5	0.1 %	No separation	No separation	56
Enzyme B	3.5	0.1 %	30	28	18

* Experiments observed for 56 hrs

rate of dissolution of the middle lamella and separation of the cells of the abscission zone. Such enzymes are conceivably present in the pectinase and enzyme B preparations used in the experiments. Evidence that enzymes effective in abscission are present in these preparations is found in the effect of IAA and methionine upon their action in causing dissolution of the middle lamella. Just as methionine and low concentration of IAA accelerate abscission, they also increase the dissolution activity of pectinase and enzyme B. Also, as high concentrations of IAA retard or prevent abscission, they prevent the action of pectinase and enzyme B in causing separation of cells.

Type of buffer salt and pH are two additional factors important in interpreting the data. Facey reported the necessity of low pH to accomplish the change from calcium pectate to pectic acid. The separation activities exemplified by enzyme B and pectinase can be illustrated only in the range of 3.5 or 4.5. No activity occurs at pH 6.2. Definite buffer effects are illustrated in table V. Carr and Ng have recently reported that several organic acids (including citric and maleic) which are commonly used as buffers, form complexes with calcium, thus removing it from the cell wall (5). This could explain the increase in separation activity of enzyme B and pectinase in the presence of the maleate and citrate buffers since the shift from calcium pectate to pectic acid would be promoted by the presence of the buffer.

The results of this investigation suggest possible roles for pectic enzymes in abscission. Factors which prevent or retard the action of PME could cause abscission by the accumulation of pectin which is soluble in water and hence ineffective as a cementing layer. Other enzymes could be involved as indicated by the in vitro separation caused by the pectinase and enzyme B preparations. A transmethylase is a probable enzyme important in yielding methyl groups for the pectic acid to pectin change. This and other enzymes which could affect the polygalacturonic chains are possibly present in the preparations used in the experiments as well as in the abscission tissue normally.

TABLE VI

EFFECTS OF VARIOUS CONCENTRATIONS OF IAA AND METHIONINE UPON SEPARATION OF CELLS OF ABSCISSION ZONE CAUSED BY PECTINASE*

CONC OF METHIONINE (M)	HRS REQUIRED FOR SEPARATION	CONC OF IAA (PPM)	HRS REQUIRED FOR SEPARATION
0.0	20	0	20
0.001	18	5	12
0.01	14	100	48
0.05	11	1,000**	No separation in 48
0.1	8		

* 0.025 % enzme solution with maleate buffer at pH 4.5
** Discolored a few hours following treatment

TABLE VII

EFFECTS OF IAA AND METHIONINE UPON SEPARATION OF CELLS BY PECTINASE AND ENZYME B PREPARATIONS

CONDITION	HRS REQUIRED FOR SEPARATION	
	PECTINASE*	ENZYME B**
No Enzyme	No separation	No separation
Enzyme	20	18
Enzyme & 0.01 M methionine	12	10
Enzyme & 5 ppm IAA	12	12
Enzyme & 0.01 M methionine and 5 ppm IAA	10	10
Ezyme & 1,000 ppm IAA***	No separation	48
Enzyme & 0.01 M methionine and 1,000 ppm IAA***	No separation in 48	48

* 0.025 % solution at pH 4.5 with 0.0025 M maleate buffer
** 0.1 % solution at pH 3.5 with 0.05 M citrate buffer
*** Discolored a few hours following treatment

Summary

PME activity has been found to be high in the abscission region of tobacco pedicels. Its activity is affected by IAA. High concentrations increase PME activity. Methionine decreases PME activity. IAA can overcome this inhibition of activity caused by methionine.

It has been possible to duplicate the separation of cells observed normally as abscission occurs by incubating tissue from the abscission zone with various pectic enzymes. The conditions which affect abscission on the plant also affect the rates of cell separation caused by these enzyme preparations.

Since IAA and methionine (which have been shown to affect abscission on intact plants) affect PME activity as well as rates of cellular separation caused by other pectic enzymes, it is possible that similar enzymes are active in the usual abscission reactions.

Two possible roles of pectic enzymes in abscission are suggested. First, conditions which prevent PME activity may cause abscission by allowing for the accumulation of pectin which is soluble in water. Or secondly, other pectic enzymes may be involved in the production of pectin or by some other chemical pathway which ultimately causes the dissolution of the middle lamella. Various pectic enzymes were shown to cause this dissolution in vitro.

Literature Cited

1. Addicott, F. T. and R. Lynch 1951. Acceleration and retardation of abscission by indole-acetic acid. Science 114: 688–689.
2. Addicott, F. T. and R. Lynch 1955. Physiology of abscission. Ann. Rev. Plant Physiol. 6: 211–238.
3. Ayres, A., J. Dingle, A. Phipps, W. W. Reid, and G. L. Solomons 1952. Enzymic degradation of pectic acid and the complex nature of polygalacturonase. Nature 170: 834–836.
4. Bryan, W. H. and E. H. Newcomb 1954. Stimulation of pectinmethylesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plantarum 7: 290–297.
5. Carr, D. J. and E. K. Ng 1959. The sequestration of calcium from preparations of wheat coleoptile cell walls. Physiol. Plantarum 12: 264–274.
6. Cormack, R. G. H. 1955. Action of pectic enzymes on surface cells of living Brassica roots. Science 122: 1019–1020.
7. Demain, A. L. and H. J. Phaff 1954. Hydrolysis of the oligogalacturonides and pectic acid by yeast polygalacturonase. Jour. Biol. Chem. 210: 381–393.
8. Demain, A. L. and H. J. Phaff 1957. Recent advances in the enzymatic hydrolysis of pectic substances. Wallerstein Lab. Communs. 20: 119–140.
9. Dingle, J., W. W. Reid, and G. L. Solomons 1953. The enzymic degradation of pectin and other polysaccharides. II. Application of the "cup-plate" assay to the estimation of enzymes. Jour. Sci. Food Agr. 4: 149–155.
10. Facey, V. 1950. Abscission of leaves in *Fraxinus americana* L. New Phytologist 49: 103–116.
11. Goodspeed, T. H. and J. N. Kendall 1916. On the partial sterility of Nicotiana hybrids made with *N. sylvestris* as a parent. Univ. Calif. (Berkeley) Publs. Botany. 5: 293–299.
12. Kendall, J. 1918. Abscission of flowers and fruits in the Solanaceae, with special reference to Nicotiana. Univ. Calif. (Berkeley) Publs. Botany 5: 347–428.
13. Kerr, T. and I. Bailey 1934. The cambium and its derivative tissues. X. Structure, optical properties, and chemical composition of the so-called middle lamella. Jour. Arnold Arboretum 15: 327–334.
14. Kertesz, Z. I. 1937. Determination of pectinmethoxylase activity. Jour. Biol. Chem. 121: 589–597.
15. Kertesz, Z. I. 1951. The Pectic Substances. Part 3. The Biochemistry of Pectic Substances. Pp. 333–398.
16. Kubart, B. 1906. Dei organische Ablosung der Korollen nebst Bemerkung über die Nolsche Tennungschichte. S.-B. Akad. Wien, Math-nat. Kl. 115.1: 1491.
17. Lloyd, F. E. 1916. Abscission of flower buds and fruit in Gossypium and its relation to environmental changes. Trans. Roy. Soc. Can. 10: 55–62.
18. Mangin, L. 1889. Sur la présence des composés pectiques dans les végétaux. Compt. rend. acad. sci., Paris. 109: 579–584.
19. McClendon, J. H. and G. F. Somers 1956. Identification of protopectinase. Plant Physiol. 31 suppl.: xliii.
20. Mohl, H. von. 1860. Ueber den Ablosungsprozess saftiger Pflanzenorgane. Bot. Zeitung. 18: 273–274.
21. Neely, W. B., C. D. Ball, C. L. Hamner, and H. M. Sell 1950. Effect of 2,4-dicholorophenoxyacetic acid on the invertase, prosphorylase, and pectinmethoxylase activity in the stems and leaves of red kidney bean plants. Plant Physiol. 25: 525–528.
22. Osborne, D. J. 1955. Acceleration of abscission by a factor produced in senescent leaves. Nature 176: 1161–1163.
23. Osborne, D. J. 1958. Changes in the distribution of pectin methylesterase across leaf abscission zones of *Phaseolus vulgaris*. Jour. Exp. Bot. 9: 446–457.
24. Schubert, E. 1952. Differentiation of the polygalacturonases of *Aspergillus niger*. Nature 169: 931–932.
25. Shoji, K., F. T. Addicott, and W. A. Swets 1951. Auxin in relation to leaf blade abscission. Plant Physiol. 26: 189–191.
26. Waygood, E. R., L. Kapica, and J. Mozie 1954. The effect of plant growth regulating substances on the production of pectin esterase by *Cladosporium herbarum* (Pers) Link. Proc. Can. Phytopath. Soc. 21: 19–24.
27. Wetmore, R. and W. Jacobs 1953. Studies on abscission: The inhibiting effect of auxin. Amer. Jour. Botany 40: 272–276.
28. Yager, R. E. Abscission zone anatomy, development, and separation in *Nicotiana tabacum*. Proc. Iowa Acad. Sci. (In Press).
29. Yager, R. E. and R. M. Muir 1958. Amino acid factor in control of abscission. Science 127: 82–83.
30. Yager, R. E. and R. M. Muir 1958. Interaction of methionine and indoleacetic acid in the control of abscission in Nicotiana. Proc. Soc. Exp. Biol. Med. 99: 321–323.

EFFECTS OF INDOLEACETIC ACID AND OTHER OXIDATION REGULATORS ON IN VITRO PEROXIDATION AND EXPERIMENTAL CONVERSION OF EUGENOL TO LIGNIN[1]

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Problems of lignin formation have long occupied the attention of investigators, however, neither the physiological nor chemical mechanisms involved in lignification is known in detail. Nevertheless, a considerable body of knowledge has accumulated through the investigations of Freudenberg, Nord, Brown, Wardrop, and others (4, 5, 6, 10, 13, 15, 23), especially on the biochemistry, histochemistry, and enzymology of lignification.

Investigations using model systems have contributed information on precursors, their transformations, (10, 17, 21) and biophysical factors in formation of lignin polymers (18). Although phenylpropane dehydrogenation polymers formed in model systems are not identical with specific lignins, they belong to this group of natural products (1, 3).

Indoleacetic acid (IAA) may regulate lignin formation. It inhibits lignification and other peroxidations in model systems (18, 19, 21) yet indirectly promotes xylem regeneration (11), presumably by inducing formation of vascular peroxidase (7, 8, 12). Antioxidant activity has been found in organic nitrogen compounds, including IAA, which possess mobile electronic systems (16, 19, 20) and provides a new experimental approach to the study of hormones and regulatory processes.

Our object is presenting evidence for the oxidation-regulating properties of several substances of biochemical interest, and the evaluation of antioxidants as possible factors in lignification.

MATERIAL AND METHODS

BIOASSAY SYSTEM: Vascular bundles of celery (*Apium graveolens*) removed as long strands, virtually free of cortical tissue, are used as a model lignifying system. The composition and properties of the synthetic lignin and its soluble derivative have been reported (18). The product approximates natural lignins (1, 3). The soluble derivative appears to consist of trimeric units containing double linked phenylpropane residues, as in the Freudenberg benzofuran structure. This work is in accord with the proposed conversion of eugenol to lignin via coniferaldehyde (1, 10).

Excised tissue was washed in cold M/15 phosphate buffer (pH 6.0) and stored at 5° C on moist filter paper until used. Excised tissues, pooled and randomized in batches of 10 to 25 gm (wet weight), could be stored and used without change in activity over periods of three to five days.

Experimental samples of 500 mg (±1 %) were shaken at 25±0.1° C for 15 hrs in 25 ml 0.2 M KH_2PO_4 (pH 4.5) containing eugenol (0.5 mM) and H_2O_2 (1.0 mM). Drifts in pH were negligible compared to the spread of the optimum plateau, pH 4 to 6.

Tissues to be analyzed for lignin were first rinsed in running water and 100 ml cold ethanol (redistilled). They were then extracted with boiling ethanol. Two 10 min extractions of tissues with 50 ml portions of alcohol, removed all soluble phloroglucinol-positive interfering substances. No dioxane-soluble product was found; hence ethanol extraction was followed by treatment of the residue with ethanol-3 % HCl which solubilizes lignins by ethanolysis (3). Pre-extracted samples were boiled 15 min in 25 ml acid ethanol, left at room temperature 30 min and the extract made up to 25 ml.

To each alcohol-extracted residue, 1 ml conc. HCl and 130 mg phloroglucinol were added rapidly with agitation, and the phloroglucinol color read at 540 mμ after 1 min (Bausch and Lomb Spectrophotometer). Color develops rapidly, reaches its maximum density in 1 min, and begins to fade after 3 min.

The phloroglucinol color in alcoholic solutions of the isolated trimer (Mol. wt. 667) (18), increased linearly with trimer concentration up to densities of about 0.5. The conversion factor was 34.5 mμ M of trimer per 0.001 density units. The ethanol-HCl extracts contain both endogenous lignin and synthetic products. Each experiment included tissue samples in buffer alone. The phloroglucinol colors in extracts from these contained only solubilized endogenous lignin and were applied as a blank correction to experimental samples incubated with eugenol and H_2O_2.

Yields of lignin in duplicate or triplicate samples from the same pooled batch of tissue agreed to within ±3 %.

PEROXIDATION OF PYROGALLOL AND EUGENOL IN VITRO: Peroxidation of pyrogallol to purpurogallin was followed photometrically at 425 mμ in a standard system (pH 4.5; 22° C; 0.005 M pyrogallol; 0.025 M H_2O_2; 1.6×10^{-8} M horseradish peroxidase, prepared using purified enzyme obtained from Nutritional Biochemicals Co. or Worthington Biochemical

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Corp.). Measurements were made at 15 sec intervals for 120 sec. For calculating inhibition the reaction rate during the first 60 sec was used. Triplicate measurements usually agreed to ±1 %. The eugenol peroxidation system was similar to that for pyrogallol, but used 0.002 M substrate and 0.005 M H_2O_2. The major products in vitro of eugenol peroxidation are dimeric substances resembling lignins in composition (18). Measurements were made at 10 sec intervals, and for calculating inhibition the overall 30 sec rate was used. (table I). Inhibitors of radical-initiated chain processes increase the induction phase of these reactions, and may be compared on this basis (2, 22). The induction time T_1, was computed graphically as shown in fig. 1.

Although coniferaldehyde forms through slow autoxidation of eugenol (10), stock and vacuum-distilled samples proved to be kinetically similar when used in vitro and in situ (with tissue). Triplicate in vitro determinations generally agreed to within 3 to 4 %.

Precautions included the use of fresh solutions of autoxidizable substances (e.g. pyrogallol, IAA, *p*-benzoquinone); spectrophotometric analysis for impurities (e.g. *p*-benzoquinone, some indoles) and recrystallization of unstable or impure solids (e.g. skatole, IAA, *p*-benzoquinone).

Results

Although the oxidant, H_2O_2, is present in a great molar excess, a number of structurally different compounds are effective inhibitors of the eugenol oxidation (table I). Oxidation in situ is generally more sensitive than in vitro. IAA, isonicotinylhydrazine, and mescaline are most notable as inhibitors, however, the activities of skatole, hydroxytyramine, and the aliphatic amines are also of interest.

The low inhibitory efficiency of indole is associated with its rapid peroxidation to an insoluble violet indigoid compound. The 5-hydroxyindoles, which were unexpectedly inefficient are readily peroxidized to 5,6-dihydroxyindoles, thence to quinones. The enhancement of lignin formation by quinones will be treated below.

Inhibitory activities are also unequal in the two in vitro systems. Eugenol oxidation is generally the more sensitive reaction, and is affected by some compounds, notably isonicotinyl-hydrazine, which are devoid of activity when pyrogallol is used as a substrate.

The two eugenol systems responded to added IAA in a qualitatively similar fashion (fig 2). The sigmoid curve described is commonly associated with inhibitors of radical-initiated reaction chains (2, 22). This comparison emphasized the sensitivity of the in situ lignin-forming system.

The existence of a chain mechanism in the enzymatic oxidation of eugenol was first suggested by its autocatalytic character (fig 1). Preliminary kinetic studies show that neither T_1 nor the post-induction rate is first order with respect to peroxidase or peroxide as they should be in a non-chain process (2, 14, 22). The exact values are yet to be determined, but are definitely not integral.

Two substances used for the study of radical-initiated polymerizations (2, 22) were tested as inhibitors of eugenol oxidation-DPPH (the radical of 1,1-diphenyl-2-(2, 4, 6-trinitrophenyl)-hydrazine and iodine. DPPH is a resonance-stabilized, violet radical which can react with a number of other radicals, including those formed from triphenylmethane, H_2O_2 (or H_2O), organic peroxides, olefins, etc. Its decolorization has been applied quantitatively, assuming that one hydrazyl radical is removed for each radical of another kind appearing. Although certain reservations have been pointed out, inhibition by DPPH is strongly indicative of the participation of radicals in the inhibition reaction. Inhibitions found at low concentrations, expressed as increase in T_1, show that

Table I

Inhibitor Efficiencies in Peroxidation of Eugenol and Pyrogallol

Catalyst / Substrate / Product / Inhibitor (M/L)	Peroxidase in situ — Eugenol — Lignin as trimer		Peroxidase in vitro — Eugenol — Dimer		Peroxidase in vitro — Pyrogallol — Purpurogallin	
	5×10^{-5}	5×10^{-4}	5×10^{-5}	5×10^{-4}	5×10^{-4}	5×10^{-3}
H_2O_2/Inhibitor (M/M)	800	80	100	10	40	4
			% Inhibition			
Indole	0	16	0	40	0	21
Skatole	21	55	25	82	0	50
IAA	71	97	53	92	41	97
5 HO-IAA	0	22	17	78	48	86
5 HO-Tryptamine	12	36	15	92	19	50
Isonicotinylhydrazine	70	99	15	97	0	0
Mescaline	70	89	15	38	0	17
HO-Tyramine	22	42	17	68	0	14
Isobutylamine	32	49	0	16	0	0
d-Glucosamine	20	38	0	10	0	0

TABLE II

EFFECTS OF DPPH AND OTHER INHIBITORS ON EUGENOL OXIDATION

INHIBITOR	CONCENTRATION (M)	H_2O_2/INHIBITOR	T_i (SEC)
None	...	...	10 ± 1
DPPH	10^{-6}	5,000	16 ± 2
	10^{-5}	500	21 ± 2
	10^{-4}	50	27 ± 3
IAA	10^{-4}	50	18 ± 1
	5×10^{-4}	10	22 ± 2
Indole	5×10^{-4}	10	14 ± 1
Skatole	5×10^{-4}	10	24 ± 2
Isonicotinyl-hydrazine	10^{-4}	50	16 ± 2
	5×10^{-4}	10	21 ± 2

DPPH is a highly efficient chain breaker (table II). Doubling of T_i is effected by approximately 10^{-5} M DPPH (500 moles H_2O_2 and 125 moles eugenol for each mole of inhibitor). Comparing inhibitor concentrations required for doubling T_i, IAA, skatole, and isonicotinylhydrazine all possess about 1/50 and indole less than 1/500 the activity of DPPH. Both DPPH and I_2 at 5×10^{-5} M inhibited lignin synthesis about 50 %, making them comparable in that system with IAA, isonicotinylhydrazine, and mescaline (table I).

ENHANCEMENT OF LIGNIN SYNTHESES BY QUINONES: Quinones may act as chain-breakers, although their inhibitory activities are sometimes secondary in importance to their chain initiating and chain transfer activities.

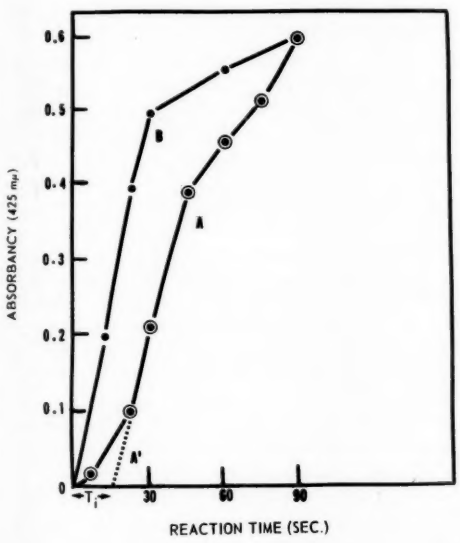
After observing that chloranil, tetrachloro-*p*-benzoquinone, effected a rapid multi-step (yellow→violet→red-brown) oxidation of eugenol in bulk and in solution, several quinones were tested as possible promoters of lignin synthesis (table III). When added to the standard in situ test system several quinones enhanced lignin synthesis substantially. Chloranil, although a far more powerful dehydrogenating agent than the others, was comparable with the parent benzoquinone and the indolic adrenochrome. Naphthoquinone was more active than other quinones as a promoter in lignin synthesis.

The behavior of quinones in the in situ assay may in turn account for the low inhibitory efficiency of 5-hydroxyindoles (table I). Autoxidation of these compounds in solution yields highly colored quinonoid intermediates, and ultimately, melanoid polymers.

TABLE III

ENHANCEMENT OF LIGNIN FORMATION BY QUINONES

QUINONE (10^{-4} M)	LIGNIN YIELD (AS SOLUBLE TRIMER) μM/500	MG TISSUE	% CONTROL
None	2.0, 2.2, 2.4, 2.4	(2.3)	100
p-benzoquinone	3.3, 3.7	(3.5)	152
Chloranil	3.1, 3.6	(3.3)	144
1,4-Naphthoquinone	5.1, 5.3	(5.2)	226
Adrenochrome	3.1, 3.7	(3.4)	148



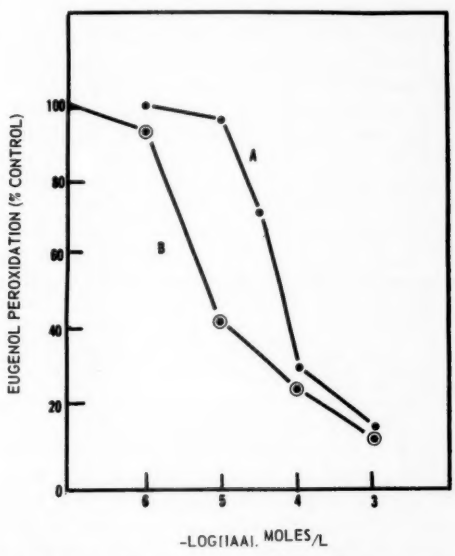


FIG. 1 (*left*). Time course of Enzymic peroxidation with two substrates. Curve A, eugenol; curve B, pyrogallol. Induction time, T_i, is obtained from A', the extrapolated linear segment of A.

FIG. 2 (*right*). Inhibition by IAA of eugenol peroxidation. Curve A, dimerization in vitro; curve B, polymerization (lignin formation) in situ.

Discussion and Conclusions

The data presented here show that IAA, other indoles, and organic N compounds behave as antioxidants in phenol oxidations of biological interest. Although antioxidant efficiency varies with the system, it is appreciable in both eugenol oxidations studied. Efficiency depends in part upon specific reactants, as is shown by comparison of two peroxidase substrates, eugenol and pyrogallol: I. pyrogallol is oxidized about 500 times faster than eugenol; II. pyrogallol oxidation is initially first order in time whereas eugenol oxidation is autocatalytic (fig 1), and exhibits other features suggesting a chain reaction; III. eugenol and its oxidation products possess far more hydrocarbon character than pyrogallol or its derivatives. Pyrogallol, therefore, should be oxidized to purpurogallin rapidly through reversible formation of short-lived radical intermediates, with the entire reaction sequence localized on the enzyme surface. In contrast, oxidation of weakly bound eugenol should proceed through reversibly formed long-lived, readily desorbed radicals, easily accessible to catalytic reducing agents or chain-terminating scavengers. The appearance in solution of a long-lived intense yellow intermediate during peroxidation of eugenol, and the inhibition of lignin formation by pyrogallol lend support to this picture (17, 18).

Among the methods used in detecting and studying of radical-initiated chain processes are I. hydroxylation of benzene. II. polymerization of added vinyl monomer, and III. inhibition by one-electron trapping agents. Of these, II can be effected by xanthine oxidase, but neither I nor II can be carried out with hydroperoxidases (14). In spite of the arguments against free-radical mechanisms in hemoprotein catalysis, there are convincing thermodynamic arguments in its favor (9). Leach (14) suggests that radical initiation is obscured by competitive OH-consuming reactions. Further, the body of chemical evidence which may be applied to the study of phenol oxidation (14, 22) demonstrates the importance of radical intermediates in these reactions.

The generation of anions from phenols undoubtedly represents an important ionic process in these reactions, but the oxidation itself almost certainly proceeds via one-electron steps (22). Thus, the controversy noted above, seems to apply to the behavior of H_2O_2-peroxidase only in the absence of phenolic substrates.

The relative efficiencies of antioxidants or scavengers in autoxidations and polymerizations vary widely (2, 22). In autoxidation of cumene, the efficiencies of substituted phenols and anilines span a nearly 500 fold range. In benzoyl peroxide-initiated vinyl acetate polymerization a series of chain-breaking nitrobenzenes and quinones of 2:1 stoichiometry (2 chains stopped inhibitor molecule) varied in efficiency by a factor of about 500. In the latter system, DPPH and I_2 were so efficient, that they could not be analyzed kinetically on a common scale with the simple aromatic inhibitors (2). Our data show that IAA, isonicotinylhydrazine, mescaline, and radical scavengers tested are of more or less comparable efficiency as inhibitors of eugenol oxidation in situ.

The H_2O_2/inhibitor ratios of 10 to 500 which were required for the doubled induction period are comparable with those observed in chemical systems using instead of H_2O_2, initiators such as benzoyl peroxide or azobisisobutyronitrile (22).

The foregoing discussion of experimental findings has necessarily placed emphasis upon chemical problems, rather than biological implications. Nevertheless, the physiological role of anti-oxidant activity warrants a brief comment. It is common knowledge that peroxidases exhibit little specificity toward their electron (or H-) donor substrates. Accordingly, the elevation of peroxidase activity under hormonal influence could in itself lead to the loss of essential thiol groups, formation of toxic quinones, hence to premature cessation of growth. In the light of existing knowledge (7, 8, 10, 11, 12), our experiments suggest that although IAA as a peroxidase-inducer is essential to vascular differentiation, its activity as an antioxidant temporarily constrains peroxidase from indiscriminate attack upon essential structures and metabolites. Such a delaying action would prolong favorable conditions for growth.

Summary

I. Indoleacetic acid and other organic nitrogen compounds inhibit oxidations involving peroxidase (in vitro) and celery vascular tissue (in situ).

II. The conversion of eugenol to lignin-like polymers in situ is especially sensitive to the antioxidants IAA, isonicotinylhydrazine, and mescaline.

III. Oxidation in vitro of eugenol is more inhibited by some compounds than oxidation of pyrogallol. This difference may reside in the differential accessibility to inhibitors of intermediate radicals formed during the oxidation of these substrates.

IV. Preliminary kinetic evidence is consistent with the chemical picture of phenolic oxidations as radical-mediated reactions. Eugenol oxidation displays some features of a radical-initiated chain reaction, whereas pyrogallol does not.

V. Free radical scavengers (chain breakers) are efficient inhibitors of eugenol oxidation in both systems. Using the H_2O_2/inhibitor ratio as an index, the inhibitory activity of the scavengers, IAA, and other compounds compares well with the activities of chain breakers and antioxidants in established oxidation and addition polymerization reactions.

VI. Quinones accelerate oxidation of eugenol in situ to lignin-like polymers, an effect which may account for the failure of autoxidizable hydroxyindoles as inhibitors in this system.

VII. The antioxidant activity of IAA may constrain the peroxidase which it induces from a premature attack upon metabolites essential to growth.

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Literature Cited

1. Adler, E. 1957. Newer views of lignin formation. Tappi 40: 294–301.
2. Bamford, C., W. Barks, A. Jenkins and P. Onyon 1958. The Kinetics Of Vinyl Polymerization By Radical Mechanisms. Academic Press, New York.
3. Brauns, F. E. 1952. The Chemistry Of Lignins. Academic Press, New York.
4. Brown, S. A. and A. C. Neish 1956. Studies of lignin biosynthesis using isotopic carbon V. Comparative studies on different plant species. Can. Jour. Biochem. Physiol. 34: 769–778.
5. Freudenberg, K., H. Reznik and D. Rosenach 1952. Das ander Verholzung beteiligte Fermentsystem. Chem. Ber. 85: 641–647.
6. Freudenberg, K., H. Reznik, W. Fuchs and M. Reichert 1955. Untersuchung uber die Entstehung des Lignins und des Holzes. Naturwiss. 42: 29–41.
7. Galston, A. W. and L. Y. Dalberg 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. Amer. Jour. Bot. 41: 373–379.
8. Galston, A. W. 1956. Some metabolic consequences of the administration of indoleacetic acid to plant cells In: The Chemistry And Mode Of Action Of Plant Growth Substances, R. L. Wain and F. Wightman, eds. Butterworth, London.
9. George, P. 1952. The specific reactions of iron in some hemoproteins. Advances in Catalysis IV: 367–428.
10. Higuchi, T. 1957. Biochemical studies of lignin formation I-III. Physiol. Plantarum 10: 356–372; 621–632; 633–648.
11. Jacobs, W. P. and Ielene Morrow 1957. A quantitative study of xylem development in the vegetative shoot apex of Coleus. Amer. Jour. Bot. 44: 823–841.
12. Jensen, W. A. 1955. Histochemical localization of peroxidase, in roots and its induction by indoleacetic acid. Plant Physiol. 30: 426–432.
13. Kratzl, K. and G. Billek 1957. Synthesis and testing of Lignin precursors. Tappi 40: 269–285.
14. Leach, S. J. 1954. The mechanism of enzymic oxidoreduction. Adv. in Enzymol. XV: 1–47.
15. Nord, F. F. and G. DeStevens 1958. Lignins and lignification In: Encyclopedia of Plant Physiology X: 389–441, W. Ruhland, ed. Springer-Verlag, Berlin.
16. Pullman, B. and A. Pullman 1958. Electron donor and acceptor properties of biologically important purines, pyrimidines, pteridines, flavins, and aromatic amino acids. Proc. Nat. Acad. Sci., U. S. 44: 1197–1202.
17. Siegel, S. 1956. The chemistry and physiology of lignin formation. Quart. Rev. Biol. 31: 1–18.
18. Siegel, S. 1959. Structural factors in polymerization: The matrix in aromatic biopolymer formation in sub-cellular particles. 1958. Soc. Gen. Physiol. Symposium. T. Hayashi, ed. Am. Physiol. Soc., Washington, D. C.
19. Siegel, S., F. Porto and P. Frost 1959. Inhibition of pyrogallol autoxidation by indole-3-acetic acid. Arch. Biochem. Biophys. (in press).
20. Siegel, S., F. Porto and P. Frost 1959. Bioregulatory activity and the nitrogen function in organic compounds: antoxidant properties and their physiological significance. Physiol. Plantarum (in press)
21. Solberg, R. and N. Higinbotham 1957. The effects of indoleacetic acid, sucrose, and eugenol on differentiation in segments of etiolated pea epicotyls. Amer. Jour. Bot. 44: 769–778.
22. Walling, C. 1957. Free Radicals In Solution. John Wiley & Sons, Inc., New York.
23. Wardrop, A. B. 1957. The phase of lignification in the differentiation of wood fibers. Tappi 40: 225–243.

SEPARATION OF MECHANISMS INITIATING CELL DIVISION AND CELL EXPANSION IN LETTUCE SEED GERMINATION[1]

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INTRODUCTION

After imbibition of water by seeds under favorable circumstances, the growth by which the embryo becomes a young seedling occurs by both expansion of cells originally present in the dormant embryo and mitotic divisions resulting in an increase in cell number (15). Germination of lettuce seed has been of special interest to plant physiologists because it can be stimulated by gibberellins, kinetin, or light (8). The sequence of the onset of cell division and elongation in germinating lettuce seed has been studied by Evenari et al (4), who found that the beginnings of mitotic activity and of cell elongation seemed to coincide in time. No mitotic activity was found in seeds prevented from germinating by withholding favorable light treatments. Their data suggested that, "The start of mitoses, the beginning of cell elongation and the protrusion of the rootlet are correlated events during germination" (4). Cell division and expansion do not begin simultaneously during germination of many other kinds of seeds. Cellular expansion precedes mitosis by many hours during germination of corn (14) or barley (2), and by several days in the broad bean (16). On the other hand, mitotic activity occurs several days before germination in pine seeds (5). Similarly, the number of cells per embryonic axis of after-ripening cherry seeds increased weeks before there was a detectable increase in axis length, which suggests that mitosis also precedes cell expansion in cherry seeds (11). Because division and expansion do not begin simultaneously during germination of many species of seeds, it is possible that lettuce might behave similarly under selected conditions. We therefore attempted to separate in time the inception of cell division from that of expansion during lettuce seed germination. The results show not only that the beginning of mitotic activity can be made either to precede or to follow cell expansion, but also that mitotic activity can be completely separated from germination. These results are of general importance in understanding the mechanisms of germination for all types of seeds.

MATERIALS AND METHODS

All experiments were performed with seeds of *Lactuca sativa*, var. New York, from the same batch of seeds previously studied in connection with light sensitivity (6) and dormancy resulting from gamma radiation (7). Cobalt 60 gamma rays were administered to the air-dry seeds as described previously (7). Seeds were then sown upon two pieces of Whatman No. 1 filter paper moistened with 5 ml of solution in covered 9 cm Petri dishes. Dishes at 26° C were under continuous white fluorescent lighting of 35 ft-c; dishes at 10° or 30° C were in darkness.

Seeds were designated as germinated with the first visible sign of rootlet expansion, namely protrusion through the seed coats. Such rootlets are hereafter called roots. Unexpanded rootlets excised from embryos of nongerminated seeds are hereafter designated radicles.

Meristems of roots or radicles were fixed in ethanol-acetic acid (3: 1), hydrolyzed in *N* HCl for 8 min at 60° C, and stained with Feulgen's reagent. The material was squashed upon slides in such a way that essentially all the cells from the apical 0.5 mm of the given root or radicle could be examined for mitotic figures.

RESULTS

It was found that, at 26° C, the beginning of mitosis and rootlet protrusion roughly coincided in time, as was observed by Evenari et al (4). Under these conditions, the time from sowing to germination was approximately 16 hrs. During germination, we seldom saw mitotic figures before protrusion, although we did observe one or two cells in division when we examined a dozen nongerminated seeds before radicle protrusion was visible. By 20 hrs (i.e., after 4 hrs growth) the roots had protruded approximately 1.5 mm through the seed coats and contained an average of several hundred mitotic figures. Experiments were designed to try to separate in time the inception of division and of expansion. For this purpose, seeds were irradiated with Co^{60} gamma rays before moistening. Although ionizing radiation affects all parts of a cell, one might expect to observe more detectable injury to mechanisms controlling cell division than to those controlling cell expansion (9, 12). Therefore seeds were given 641 kr of gamma radiation and sown on 10^{-5} M kinetin at 26° C. Kinetin was used to reverse the inhibition of germination percentages effected by irradiation with 641 kr (7). Table I illustrates that, when seeds were given 641 kr gamma radiation and sown on kinetin, cell expansion (as indicated by rootlet protrusion) and mitosis were both greatly de-

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[2] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

layed. However, the beginning of mitosis was delayed more than that of cell expansion. No mitosis was detected in meristems from expanded roots after two, three, and four days. Even more striking is the fact that mitosis was not observed in any of the roots examined until two days after germination was first observed. Micronuclei were abundant in interphase cells from root tips of New York lettuce seeds after these seeds were given 214 kr from the same gamma source (7). However, all root meristems that lacked mitotic figures also lacked micronuclei. Whereas the absence of mitotic figures in the entire root tip shows that no cells were undergoing mitosis at the time of fixation, the absence of micronuclei further suggests that no mitotic divisions occurred before fixation. For comparison, when unirradiated seeds were germinated in water at 26° C and roots had grown 1.5 mm (20 hrs after sowing, i.e., approximately 4 hrs after rootlet protrusion) 55, 60, 233, 348, 435, 524, 540, 592, and 692 figures were found in nine root tips. Although we thus conclude that gamma irradiation permits cellular expansion to proceed before mitosis begins (table I), it is clear from our previous studies that no dose of gamma radiation can be found that permits germination that is not eventually accompanied by mitotic activity (7).

The results described in the last paragraph raise the question as to whether other treatments might give converse results, namely, mitotic activity before germination. Several investigators have used mannitol to prevent cellular expansion by interfering with water uptake (1, 3). Consequently, one might expect that mannitol would interfere more with cell expansion than with cell division. As was found with irradiated seeds in kinetin (table I), seeds germinated much more slowly in the presence of various concentrations of mannitol at 26° C than did untreated seeds in water (table II). In contrast to the results with gamma radiation, however, mitosis occurred before protrusion when seeds were in mannitol, since mitotic figures were frequently found in nongerminated seeds (table II). For seeds in 0.5 M mannitol, mitotic figures were detected after one day, even though germination was not detected until a week later. Only 2.5 % of the seeds in 0.5 M mannitol had germinated after 16 days, and no further germination occurred thereafter. The presence of mitotic activity after one day was compared with the percentage germination after 16 days by calculating χ^2 by the method of the 2×2 table (13). The corresponding value of p was 1.2×10^{-3}. Thus we can conclude not only that mannitol delays germination more than it delays mitosis, but that it can prevent germination of seeds that nonetheless undergo mitotic activity.

Further experiments were conducted to see if treatments other than gamma radiation or mannitol also could alter the sequence of the beginnings of mitosis and germination. Results parallel to those found with irradiated seeds were found for untreated

TABLE I

TIME COURSE OF GERMINATION AND MITOTIC ACTIVITY IN SEEDS GIVEN 641 KR OF GAMMA RAYS AND SOWN ON 10^{-5} M KINETIN AT 26° C

DAYS AFTER SOWING	% GERMINATION OF ALL SEEDS*	GERMINATED POPULATION ONLY	
		AVERAGE ROOT LENGTH (MM)	FRACTION OF ROOTS WITH MITOTIC FIGURES**
1	0	...	...
2	6	1.00	0/4
3	50	1.66	0/10
4	68	2.72	5/10

* Germination percentages for each day were calculated from independent samples of 100 seeds each.

** Number of roots with one or more mitotic figures/number of roots examined.

TABLE II

TIME COURSE OF GERMINATION AND MITOTIC ACTIVITY IN SEEDS SOWN ON SOLUTIONS OF MANNITOL AT 26° C

DAYS AFTER SOWING	MANNITOL CONCENTRATION					
	0.3 M		0.4 M		0.5 M	
	% GERMINATION*	No. OF 10 RADICLES WITH MITOTIC FIGURES**	% GERMINATION*	No. OF 10 RADICLES WITH MITOTIC FIGURES**	% GERMINATION*	No. OF 10 RADICLES WITH MITOTIC FIGURES**
1	0.0	3	0.0	4	0.0	3
2	0.3	8	0.0	6	0.0	2
3	2.0	6	0.0	4	0.0	2
4	4.6	1	0.3	0	0.0	0
5	9.1	2	0.6	2	0.0	0
6	9.3	...	1.0	...	0.0	...
7	9.3	3	1.3	0	0.0	1
8	10.3	...	1.8	...	0.0	...
10	14.3	3	3.3	4	0.5	0
16	29.3	...	28.3	...	2.5	...

* Calculated from 100 to 350 seeds.

** Radicles selected only from the populations of seeds that had not germinated at times indicated. Data refer to radicles with one or more mitotic figures.

TABLE III

TIME COURSE OF GERMINATION AND MITOTIC ACTIVITY AT 10° C

DAYS AFTER SOWING	% GERMINATION OF ALL SEEDS*	GERMINATED POPULATION ONLY: AVERAGE ROOT LENGTH (MM)	GERMINATED POPULATION ONLY: FRACTION OF ROOTS WITH MITOTIC FIGURES**
3	0	...	...
4	1	0.5	0/1
5	44	1.5	4/20***
6	71	2	8/11
7	83	2	9/11

* Germination percentages for each day were calculated from independent samples of 88 to 92 seeds.

** Number of roots with mitotic figures/number of roots examined.

*** See table IV for frequency distribution of mitotic figures.

seeds germinated at 10° C (table III). Again, the times of cell division and of germination were greatly delayed in comparison to the times observed with untreated seeds germinating at 26° C. Beginning of mitosis seemed, however, to be more delayed than the beginning of germination. Thus after five days, 16 of 20 protruded roots had no mitotic figures. Of the four root tips that did have mitotic figures after five days, three had only one figure, and one had two figures. These data should be compared with the hundreds of mitotic figures found in roots of the same length when seeds were germinated at 26° C (see data in first paragraph of this section). The frequency distribution of mitotic figures among the 20 root tips examined after five days at 10° C is shown in table IV. The frequencies expected from a Poisson distribution with the same mean number of mitotic figures per root were calculated for comparison. The agreement between the observed frequencies of mitotic figures per tip and the frequencies calculated from a Poisson distribution suggests that soon after rootlets have protruded, mitosis occurs randomly.

Experiments were performed also at 30° C, just on the upper limit of the temperature range that permits germination. Table V summarizes an experiment in which separate dishes of seeds at 30° C were examined after various times for germination percentages, and radicles were excised from 20 of the nongerminated seeds and examined for mitotic activity. It has been our experience that, at this temperature, all seeds that will germinate do so within one or two days. Consequently, we do not consider the 5 % germination after eight days to be a biologically significant increase over the germination percentages at earlier times. Mitotic activity occurs in nongerminated seeds at 30° C (table V) just as it does in mannitol at 26° C (table II). The appearance of mitotic activity after one day was compared with the percentage germination after eight days by the method described. The difference was significant at the level of 10^{-4}. Thus we can conclude that the 30° C temperature, like treatment with 0.5 M mannitol at 26° C, prevents germination of seeds that undergo mitotic activity. Figure 1 illustrates the frequency distribution of mitotic figures among the same groups of 20 radicles listed in table V. The data in figure 1 suggest more readily than does table V that the mitotic activity in the nongerminating seeds tends to decrease with increasing time. More important, however, figure 1 shows a marked deviation of the observed distributions from those calculated for the corresponding Poisson distributions with the same mean number of mitotic figures per group of radicles. The observed frequencies are higher than the calculated frequencies for those radicles having no mitotic figures, as well as for classes having many more mitotic figures than expected from the Poisson distributions. This type of deviation from randomness suggests a tendency for an all-or-none characteristic (i.e., no mitoses vs. many) in the occurrence of mitoses in nongerminated seeds. Such an all-or-none characteristic is analogous to the normal germination process in lettuce, for which it is often found that under a given set of conditions a single batch of seeds will consist almost entirely of two populations: one population in which none of the seeds germinate, and one population in which all of the seeds germinate and become seedlings capable of growth to maturity.

TABLE IV

DISTRIBUTION OF MITOTIC FIGURES AMONG ROOT TIPS FROM GERMINATED SEEDS 5 DAYS AFTER SOWING AT 10° C

MITOTIC FIGURES PER ROOT TIP	OBSERVED FREQUENCY	FREQUENCY EXPECTED FROM POISSON DISTRIBUTION
0	16	15.58
1	3	3.89
2	1	0.49
3	0	0.04
Total	20	20.00

TABLE V

TIME COURSE OF GERMINATION AND MITOTIC ACTIVITY IN SEEDS AT 30° C

DAYS AFTER SOWING	% GERMINATION*	NO. OF 20 RADICLES WITH MITOTIC FIGURES**
1	0.8	13
2	0.0	13
3	3.3	6
5	0.8	4
8	5.0	2

* Percentage of 120 seeds

** Radicles selected from nongerminated seeds. See fig 1 for frequency distribution of mitotic figures.

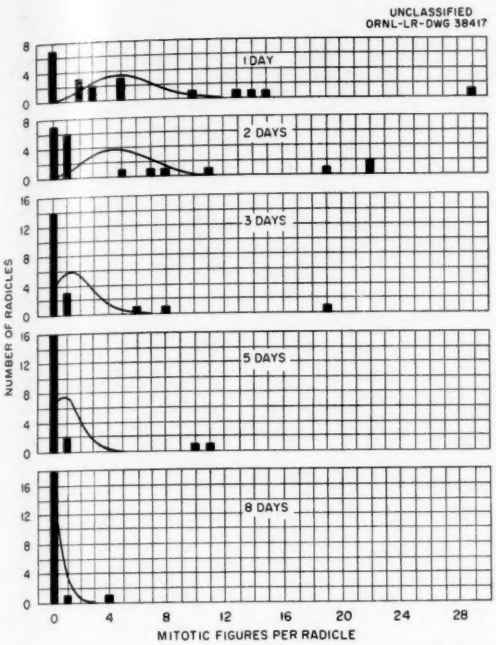

FIG. 1. Distribution of mitotic figures among radicles excised from nongerminated seeds at 30° C. Bars represent observed frequencies. Curves connect points calculated from Poisson distributions.

At 40° C, seeds neither germinated nor did they undergo any detectable mitotic activity.

DISCUSSION

These results indicate that the beginning of cell division and the beginning of cell expansion are not correlated events during germination. Gamma radiation or low temperatures delay the beginning of cell division until after cell expansion has resulted in rootlet protrusion. Whereas root tips from irradiated seeds germinated at 26° C or from unirradiated seeds germinated at 10° C had no mitotic figures, roots of comparable length from untreated seeds germinated at 26° C had several hundred mitotic figures. Conversely, mannitol or high temperatures tend to permit some cell division before cell expansion can be detected by rootlet protusion. At 30° C in water or 26° C in 0.5 M mannitol, mitoses occur in seeds that do not subsequently germinate under these conditions. It is pertinent that these seeds that can be prevented from germinating by holding at 30° C do germinate when transferred back to 26° C (Haber, unpublished). Consequently, the failure of seeds having mitotic activity at 30° C to germinate cannot be explained by saying that loss of viability intervened between the time that mitotic activity began and the time that expansion would have begun. Moreover, the appearance of mitotic figures in nongerminating seeds at 30° C cannot be explained by saying that mitoses were initiated but not completed, since the number of mitotic figures per radicle tended to decrease with time (fig 1).

The data of Evenari et al (4) apparently resulted from the use of conditions under which the beginnings of cell division and cell elongation happened to coincide. In comparing their results with our own, another factor to consider is the different cytological techniques used. In our studies, we used squashed preparations, which enabled us to examine many cells of a root tip including essentially all the thousands of cells in the meristematic region. Evenari et al examined 12-μ-thick longitudinal sections instead of the whole root tip. It should be emphasized that our studies are concerned with the initiation, and not the extent, of mitosis. The data do show that the extent of cell division is generally greater after rootlet protrusion than before protrusion. Such increased mitotic activity, however, is probably a result of growth, since it is neither correlated with nor necessary for the initiation of cell expansion. Thus our results are an extension of, and not necessarily in contradiction to, the earlier work of Evenari et al (4).

Protrusion of the rootlet during lettuce seed germination is caused by cell expansion, whereas mitotic division contributes little or nothing. This conclusion follows from the fact that the radicle of a nongerminated seed can have as many as 29 mitotic figures at one time (fig 1), whereas a germinated seed can have a root that has expanded more than 1.6 mm through the seed coat without undergoing any mitoses (as indicated by the absence of mitotic figures and micronuclei after gamma irradiation, table I). This conclusion is consistent with the facts that corn (14), barley (2), and broad bean (16) seeds normally undergo rootlet protrusion before mitosis begins, whereas pine (5) and, presumably, cherry (11) seeds undergo mitotic activity before rootlet protrusion. Thus the mechanism of rootlet protrusion in seeds is parallel to the mechanism of root extension in mature plants, which is caused chiefly by expansion of cells in the zone of elongation. Mitotic activity functions in root elongation indirectly, i.e., by proliferating new cells which, in turn, are capable of contributing to root extension by their own expansion.

Although gamma radiation can cause germination to precede mitotic activity, we have shown that any dose of gamma radiation that will permit lettuce seed germination also will permit mitotic activity (7). Consistent with this is our present observation that at 10° C, where germination also precedes mitotic activity, mitoses seemed to occur randomly among protruded roots (table IV). By way of contrast, at 30° in water or at 26° C in 0.5 M mannitol, mitotic activity could be completely separated from germination, since seeds undergoing mitotic activity did not germinate at all. Analysis of the frequency distribution of mitoses among radicles of nongerminating seed at 30° C showed that mitoses were not occurring ran-

domly, but tended to conform to an all-or-none pattern (fig 1). This suggests that there are mechanisms preventing mitotic activity in some and permitting mitotic activity in other nongerminating seeds at 30° C. This is analogous to the findings that there are mechanisms that prevent cell expansion in some and permit cell expansion in other seeds during normal germination at 22 to 26° C. Clearly, the mechanisms by which mitotic activity is blocked must be different from the mechanisms by which cell expansion is blocked, since the seeds with mitotic activity at 30° C (fig 1) were nongerminating.

These results also have implications for studies of the mechanisms of action of germination stimulators. Since rootlet protrusion results from cell expansion and since kinetin stimulates germination of gamma-irradiated seeds under conditions where cell expansion precedes mitosis by several days (7, table I), it is obvious that the *germination-stimulating* activity of kinetin must be attributed to an initiation of cell expansion and not to any possible stimulation of cell division. These considerations are especially interesting because kinetin was first studied in connection with its activity as a cell-division factor (10). Presumably other germination-stimulating agents must similarly function by stimulating cell expansion (7). This last statement is not intended to suggest that the mechanisms of action of different germination stimulators are identical, since we have previously shown that effects of gibberellic acid, kinetin, and red light could be separated from one another (8). Nor do we wish to imply that germination-stimulating agents may not also affect mitotic activity in seeds apart from their effects on cell expansion. An interesting problem for future research will be to determine whether the light and chemicals that stimulate normal germination can stimulate mitotic activity in nongerminating seeds.

The different sequences of the inceptions of cell division and cell expansion at extremely high and extremely low temperatures indicate different physiological properties of germination at the different temperatures. These considerations are reminiscent of earlier work from our laboratory, performed with Grand Rapids lettuce seeds, which showed that kinetin stimulates germination at superoptimal temperatures where gibberellic acid is ineffective, whereas the reverse is true at low temperatures (8).

Summary

I. At 26° C lettuce seed germinates rapidly, and the beginnings of cell division and cell expansion roughly coincide in time.

II. When gamma-irradiated seeds are sown on a solution of kinetin at 26° C, germination, although greatly delayed compared to that of unirradiated controls, precedes cell division by several days.

III. Results similar to those with irradiated seeds were found with unirradiated seeds germinating at 10° C. The distribution of mitoses among root tips of newly germinated seeds at 10° C suggested that mitoses occurred randomly among them.

IV. When seeds were germinated at 26° C in solutions of mannitol, cell divisions preceded germination by many days. High concentrations of mannitol prevented germination of seeds that nevertheless underwent mitotic activity.

V. At 30° C, only a very small percentage of seeds germinate. Most seeds, however, undergo cell division. The distribution of mitoses among radicle meristems of nongerminating seeds showed that mitoses were not occurring randomly among the unexpanded radicles. Rather, there was a tendency for an all-or-none characteristic in the occurrence of mitoses within individual radicles. As time progressed, the mitotic activity in these nongerminating seeds diminished.

These results indicate that A. The initiation of cell expansion and that of cell division are controlled by separate mechanisms during germination. B. Rootlet protrusion during germination results from cellular expansion, whereas cell division plays little or no role. C. The mechanisms by which kinetin and other agents stimulate lettuce seed germination are related to the initiation of cellular expansion and not to cell division.

Literature Cited

1. Burström, H. 1954. Studies on growth and metabolism of roots. XI. The influence of auxin and coumarin derivatives on the cell wall. Physiol. Plantarum. 7: 548–559.
2. Caldecott, R. S. and L. Smith 1952. A study of X-ray-induced chromosomal aberrations in barley. Cytologia (Japan) 17: 224–242.
3. Cleland, R. and J. Bonner 1956. The residual effect of auxin on the cell wall. Plant Physiol. 31: 350–354.
4. Evenari, M., S. Klein, H. Anchori and N. Feinbrun 1957. The beginning of cell division and cell elongation in germinating lettuce seed. Bull. Res. Council Israel 6 D: 33–37.
5. Goo, M. 1952. When cell division begins in the germinating seeds of *Pinus thunbergii* Parl. Jour. Jap. Forestry Soc. 34: 3–4.
6. Haber, A. H. 1959. Rendering the germination of light-insensitive lettuce seeds sensitive to light. Physiol. Plantarum. 12: 456–464.
7. Haber, A. H. and H. J. Luippold 1959. Dormancy resulting from gamma-irradiation of lettuce seed. Int. Jour. Radiation Biol. 1: 317–327.
8. Haber, A. H. and N. E. Tolbert 1959. Effects of gibberellic acid, kinetin, and light on the germination of lettuce seed. In: Photoperiodism and Related Phenomena in Plants and Animals. R. B. Withrow, ed. AAAS, Washington, D. C. Pp. 197–206.
9. Haskins, F., M. Davidson and R. Beers 1958. Influence of seed irradiation with X-rays and thermal neutrons upon cell size and mitotic activity in root tips of maize. Amer. Naturalist 92: 365–369.

10. Miller, C., F. Skoog, F. Okumura, M. von Saltza and F. Strong 1956. Isolation, structure and synthesis of kinetin, a substance promoting cell division. Jour. Amer. Chem. Soc. 78: 1375–1380.
11. Pollock, B. M. and H. O. Olney 1959. Studies of the rest period. I. Growth, translocation, and respiratory changes in the embryonic organs of the after-ripening cherry seed. Plant Physiol. 34: 131–142.
12. Schwartz, D. and C. E. Bay 1956. Further studies on the reversal in the seedling height dose curve at very high levels of ionizing radiations. Amer. Naturalist 90: 323–327.
13. Snedecor, G. W. 1956. Statistical Methods. (5th ed.) Iowa State College Press, Ames
14. Toole, E. H. 1924. The transformations and course of development of germinating maize. Amer. Jour. Bot. 11: 325–350.
15. Toole, E. H., S. B. Hendricks, H. A. Borthwick and V. K. Toole 1956. Physiology of seed germination. Ann. Rev. Plant Physiol. 7: 299–324.
16. Wolff, S. 1954. Some aspects of the chemical protection against radiation damage to *Vicia faba* chromosomes. Genetics 39: 356–364.

INHIBITION OF PHOTOPERIODIC INDUCTION BY 5-FLUOROURACIL[1, 2]

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In this paper it will be shown that photoperiodic induction of the cocklebur, a short day plant, is inhibited by the pyrimidine 5-fluorouracil (5-FU). The studies of other workers have shown that 5-FU inhibits the growth of various kinds of cells and tissues by suppressing the formation of thymidine, and that application of thymidine relieves the inhibitory effects of 5-FU. In these cases 5-FU is an inhibitor of DNA synthesis (2, 4, 5). In other cases, however, 5-FU inhibits RNA synthesis. Thus 5-FU inhibits the production of tobacco mosaic viral RNA by tobacco leaves (1). This inhibition, which is relieved neither by thymidine nor by uracil, is associated with incorporation of 5-FU into the viral RNA (3). Inhibition of photoperiodic induction by 5-FU is not reversed by either thymidine or uracil but is reversed by orotic acid (6) which is known to be an intermediate in the biogenesis both of uridine and cytidine and of deoxycytidine and thymidine. Reversal of 5-FU inhibition by orotic acid suggests the hypothesis that the inhibition is related in some way to suppression of nucleic acid metabolism.

The experiments here reported were further designed to determine which component process of floral induction is inhibited by 5-FU. It will be shown that one effect of 5-FU is upon the inductive act by which vegetative buds are so changed that they subsequently develop into floral primordia.

Methods

The general procedures used in this investigation, which have been described in detail elsewhere (8, 9), were as follows: Cocklebur plants (*Xanthium pennsylvanicum* Wall.[3]) were grown from seed of our standard inbred line. They were maintained in the vegetative condition in the greenhouse by the use of supplementary low intensity light to extend the natural day length to approximately 20 hours per day. The plants were used 60 days or more after planting and, therefore, after the appearance of the first typically mature leaves. One day before each experiment, the plants to be used were sorted according to size of the half-expanded (most sensitive) leaf. One leaf above the half-expanded leaf and all leaves below this were removed from each plant. The plants were then distributed into groups; all groups contained equal representations of each size class of leaf. In general, one group of 10 to 20 plants was used for each treatment of each experiment; each experiment was repeated three to eight times as noted below.

Chemical treatments were applied by dipping the leaf or tip or both into a solution of the chemical in question. Dipping of the apical bud alone in this way results in the retention by the bud of approximately 0.05 cc of treatment solution. Treatment of the

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[2] Supported in part by grants from the Frasch Foundation, National Science Foundation, and the Colorado State University Research Foundation. This work was reported at the Annual Meeting, American Society of Plant Physiologists, Indiana University, August, 1958.

[3] Plants used have been classified by H. D. Harrington, taxonomist, Colorado State University, using recent manuals of the Illinois region from which the plants originated, as *Xanthium italicum* Moretti. We will continue to use *X. pennsylvanicum* Wall. until the taxonomy of Xanthium has been clarified. Specimens of the plants typical of those used in our experiments are on file in the Colorado State University Herbarium.

TABLE I

DATA RELATING TO EXPERIMENTAL CONDITIONS FOR RESULTS SHOWN IN FIGS 1 TO 6

FIG NO.	EXPERIMENT NO.	DATE*	PLANTS/ TREATMENT	LEAF LEFT ON PLANT**	NO. SIMILAR EXPERIMENTS PERFORMED
1	C-93	2/12/58	10	S-#3	5
2	C-132	4/16/59	20	T-#3	8
3	C-104	5/5/58	13	T-#3	2
4	C-99	3/27/58	15	T-#3	3
5	C-109	5/24/58	10	T-#3	4
6	C-112	7/17/58	20	T-#3	3

* Date plants were placed in cabinets.
** Numbers refer to length of leaf midrib as follows: S-#3, 5.9 to 7.7 cm; T-#3, 6.9 to 8.5 cm.

leaf alone results in the retention of approximately 0.49 cc of solution. The amount of chemical actually transferred to the plant from a given treatment solution is therefore approximately ten times greater when treatment is applied to the leaf than when treatment is applied to the bud. All treatment solutions contained approximately six drops of Tween 20 per liter.

With the exception of the critical dark period experiment (fig 3), a single 16 hour dark period was used for induction. At the end of the dark period, the plants were returned to long day conditions in the greenhouse. Nine days later the apical buds were dissected, examined under a microscope, and classified according to the series of floral stages previously described (7). Floral stage, as used here, is a measure of rate of development and degree of induction of the cocklebur plant. The experimental details of date, leaf size, number of plants per treatment and number of replications of the experiment are given in table I for all experiments here reported.

Standard errors were calculated for the floral stage estimate of each treatment. From the mean treatment standard error, the minimal difference between two treatments required for significance at the 5 % level was calculated and is included in the data for each experiment.

RESULTS

In preliminary experiments it was established that thiouracil and 2,6-diaminopurine are effective in inhibition of photoperiodic induction of the cocklebur. 5-hydroxyuracil (up to 2×10^{-3} M) and 5-mercaptouracil (up to 1×10^{-2} M), kindly supplied by Dr. Thomas J. Bardos, Armour Laboratories, were ineffective in the concentrations used. 5-Fluorouracil (5-FU) kindly supplied by Hoffman-La Roche, Nutley, N. J., is, however, highly effective in this function.

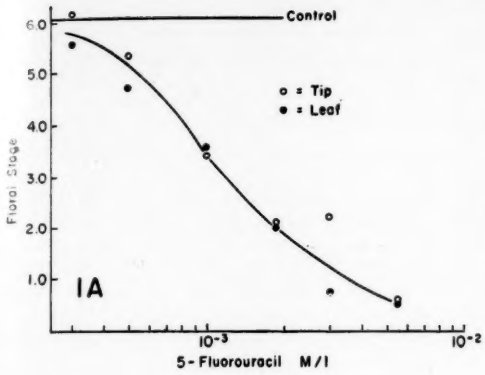
In experiments of which that of figure 1 is typical, 5-FU was applied to cocklebur plants over a wide range of concentrations. Half inhibition of photoperiodic induction, as measured by rate of floral development, is elicited by approximately 10^{-3} M 5-FU. Figure 1 includes data on application of 5-FU to leaves as well as to apical buds alone. In this experiment the concentration of 5-FU which must be ap-

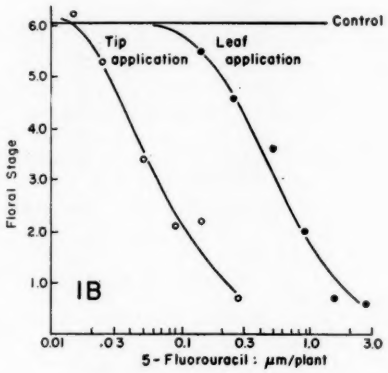
FIG. 1. Inhibition of photoperiodic induction of cocklebur as a function of concentration of applied 5-fluorouracil (5-FU) concentration (A) and amount (B). A single application of inhibitor was made either to the leaf alone or to the apical bud alone. Stage of floral development measured 9 days after the end of the 16 hour inductive dark period. Minimal difference between two treatments required for significance at the 5 % level of probability is 1.41 floral stage units.

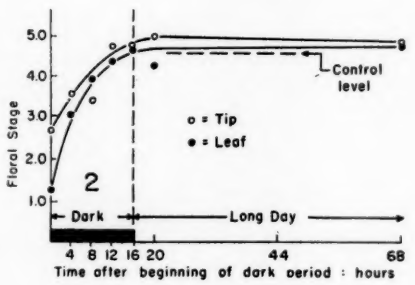
FIG. 2. Inhibition of photoperiodic induction of cocklebur by 5-FU (2×10^{-3} M) as a function of time of application. Stage of floral development measured 9 days after the end of the 16 hour inductive dark period. Minimal difference between two treatments required for significance at the 5 % level of probability is 0.98 floral stage units.

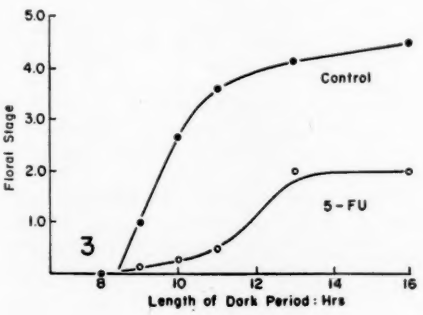
FIG. 3. Inhibition of floral induction of cocklebur by 5-FU (2.0×10^{-3} M) as a function of length of the inductive dark period. A single application of 5-FU was made to both leaf and bud at the start of the inductive dark period in each instance. Stage of floral development measured 9 days after the end of the inductive dark period. Minimal difference between two treatments required for significance at the 5 % level of probability is 0.98 floral stage units.

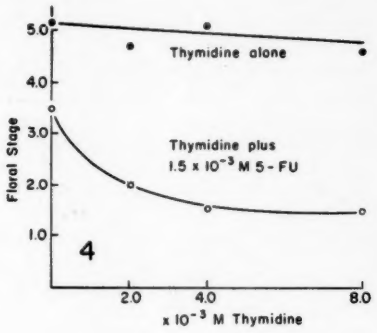
FIGS. 4, 5, and 6. Floral induction of cocklebur as a function of concentration of applied thymidine (fig 4), uracil (fig 5), or orotic acid (fig 6) and in the presence or absence of applied 5-FU. In the experiments of figures 4 and 6 applications were to both bud and leaf, in that of figure 5, to leaf or bud as indicated. Minimum differences between two treatments required for significance at the 5 % level of probability 1.12 (fig 4), 1.15 (fig 5) and 0.98 (fig 6) floral stage units.

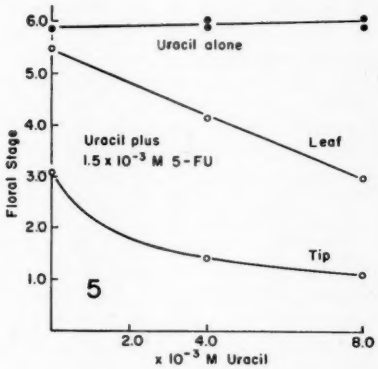

Control
o = Tip
● = Leaf
Floral Stage
1A
10⁻³
10⁻²
5-Fluorouracil M/l

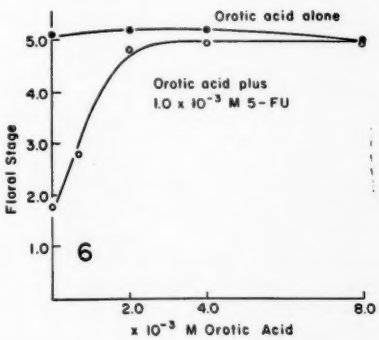

Control
Tip application
Leaf application
Floral Stage
1B
0.01
.03
0.1
0.3
1.0
3.0
5-Fluorouracil : μm/plant


Control level
o = Tip
● = Leaf
Floral Stage
2
Dark
Long Day
4 8 12 16 20 44 68
Time after beginning of dark period : hours


Control
5-FU
Floral Stage
3
8 10 12 14 16
Length of Dark Period : Hrs


Thymidine alone
Thymidine plus
1.5 x 10⁻³ M 5-FU
Floral Stage
4
2.0 4.0 8.0
x 10⁻³ M Thymidine


Uracil alone
Uracil plus
1.5 x 10⁻³ M 5-FU
Leaf
Tip
Floral Stage
5
2.0 4.0 8.0
x 10⁻³ M Uracil


Orotic acid alone
Orotic acid plus
1.0 x 10⁻³ M 5-FU
Floral Stage
6
2.0 4.0 8.0
x 10⁻³ M Orotic Acid

plied to buds to elicit half maximal inhibition is the same as the corresponding concentration for leaf application. The amount of solution and hence of 5-FU applied to the bud is, as noted above, smaller than that applied to the leaf. The 0.05 cc of 10^{-3} M 5-FU solution per bud required to elicit half maximal inhibition, contains 0.05 μ moles of 5-FU. The 0.49 cc of 10^{-3} M 5-FU solution per leaf required to elicit the same effect contains 0.49 μ moles of 5-FU. Thus less 5-FU is required to elicit a given degree of inhibition if the substance is applied to the bud than if it is applied to the leaf.

The greater effectiveness of 5-FU in inhibition of photoperiodic induction, when applied to buds rather than to leaves, was in occasional experiments even more striking than in that of figure 1. In the experiment of figure 5, for example, the application of 5-FU to apical buds produced substantial inhibition in a concentration which was almost ineffective when applied to leaves. The reasons for these variations are unknown.

In the experiment of figure 2, 5-FU was applied to plants at the beginning of, during, or after the end of the inductive dark period. Floral stage, as measured nine days after the inductive dark period, is plotted in figure 2 as a function of time after the beginning of induction. In the experiment of figure 2, as well as in seven others not here presented, 5-FU was most effective when applied at the beginning of the dark period and virtually ineffective when applied at the end of the dark period. This is true for 5-FU application to the tip as well as for application of the material to the leaf. Plants treated with an appropriate concentration of 5-FU at the beginning of the inductive dark period not only do not subsequently flower but continue to grow vegetatively. A single application of 5-FU does not therefore exert any lasting and general inhibition of growth.

A substance which inhibits photoperiodic induction only when it is applied during the dark period may influence either the time-measuring reactions of the dark period or the processes which take place during the inductive dark period but after the expiration of the critical night length (8). Figure 3 shows the results of an experiment designed to determine the effects of 5-FU on the time-measuring phase of the inductive process. Individual groups of cocklebur plants were given single dark periods varying in length from 8 to 16 hours. In figure 3, floral stage 9 days after induction is plotted as a function of length of the dark period. It is clear that in the untreated control plants, flowering took place only when the dark period exceeded approximately 8.5 hours. The same is true for 5-FU treated plants. It is evident, therefore, that 5-FU does not affect the critical dark length and hence does not affect the time-measuring reactions of photoperiodic induction. The data of figure 3 show, however, that 5-FU decreases the effectiveness of the processes which go on in dark periods longer than the critical and which lead to flowering. We may tentatively conclude then that the primary effect of 5-FU upon the act of induction is somehow or other concerned with the floral stimulus which is produced during the inductive dark period.

The data of figures 4, 5, and 6 concern the reversibility of 5-FU inhibition by the nucleotide precursors thymidine, uracil, and orotic acid. It is clear that neither thymidine nor uracil applications reverse 5-FU inhibition of flowering. Indeed, in these experiments combinations of thymidine or uracil with 5-FU caused a greater inhibition of flowering than did 5-FU alone. Neither thymidine, uracil, nor orotic acid by themselves exhibited any effect upon flowering. Orotic acid application does, however, completely overcome the inhibitory effect of 5-FU on flowering (fig 6). This result suggests that the inhibitory effect of 5-FU on flowering may be exerted upon some aspect of nucleotide metabolism.

In all experiments in which 5-FU was applied to cocklebur plants, it was noted that the inhibitor, in addition to suppressing photoperiodic induction, caused a reduction in rate of expansion of the young leaves and elicited some wrinkling of these leaves. It is interesting that orotic acid, which reverses the effect of 5-FU upon flowering, does not reverse the vegetative effects of 5-FU. Thus the effects of 5-FU upon flowering may be separated from those upon vegetative growth.

Discussion

Since 5-FU inhibits flowering effectively only when applied during the single inductive dark period (fig 2) and does not influence the length of the critical night (fig 3), we may tentatively conclude that the effect of 5-FU is upon synthesis or effectiveness of the products of the inductive dark period. From the known interference of 5-FU in nucleic acid metabolism and the inhibition of photoperiodic induction by this compound, we infer that nucleic acid metabolism is involved in the inductive process.

The fact that 5-FU is an effective inhibitor of induction when applied to the apical bud, indicates that an integral part of the inductive process takes place in the bud during the inductive dark period. This has not previously been recognized. It has heretofore been assumed, on good grounds, that the dark reactions are confined to the tissues of the leaf. The present experiments suggest, however, that some portion of the inductive process involving nucleic acid metabolism takes place in the bud during the period of hormone synthesis in the leaf.

Summary

I. Photoperiodic induction of *Xanthium pennsylvanicum*, the cocklebur, is inhibited by application of 5-fluorouracil.

II. A given amount of 5-fluorouracil is in general more effective in inhibiting photoperiodic induction when applied to the buds than when applied to the leaves, the known perceptor organ in photoperiodic induction.

III. 5-Fluorouracil is fully effective in inhibiting photoperiodic induction only if applied during the inductive dark period. This is true even of 5-fluorouracil application to the apical bud. It appears, therefore, that something essential to induction takes place in the bud during the exposure of the leaf to an inductive dark period.

IV. 5-Fluorouracil inhibition of photoperiodic induction is reversed by applying orotic acid. The hypothesis is suggested that photoperiodic induction involves nucleotide metabolism, possibly nucleic acid synthesis.

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Literature Cited

1. Davern, C. I. and J. Bonner 1958. The influence of 5-fluorouracil on tobacco mosaic virus production in tobacco leaf discs. Biochem. Biophys. Acta 29: 205–206.
2. Eidinoff, M., J. E. Kroll, and D. Klein 1957. Effect of 5-fluorouracil on the incorporation of precursors into nucleic acid pyrimidines. Arch. Biochem. Biophys. 71: 274–275.
3. Gordon, M. and M. Staehelin 1958. Incorporation of 5-fluorouracil into the nucleic acid of tobacco mosaic virus. Jour. Amer. Chem. Soc. 80: 2340–2341.
4. Heidelberger, C., L. Bosch, N. K. Chaudhuri, and P. B. Danneberg 1957a. Mechanism of action of 5-fluoropyrimidines. Fed. Proc. 16: 194.
5. Heidelberger, C., N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. Schnitzer, E. Pleven, and J. Scheiner 1957b. Fluorinated pyrimidines, a new class of tumor inhibitory compounds. Nature 179: 663–666.
6. Mitchell, H. K., M. Houlahan, and J. Nyc 1948. The accumulation of orotic acid by a pyrimidineless mutant of Neurospora. Jour. Biol. Chem. 172: 525–529.
7. Salisbury, F. 1955. The dual role of auxin in flowering. Plant Physiol. 30: 327–334.
8. Salisbury, F. 1957. Growth regulators and flowering. I. Survey methods. Plant Physiol. 32: 600–608.
9. Salisbury, F. 1959. Growth regulators and flowering. II. Cobaltous ion. Plant Physiol. 34: 598–604.

EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID APPLICATION ON ACTIVITY AND COMPOSITION OF MITOCHONDRIA FROM SOYBEANS [1, 2]

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It can be assumed that mitochondria possess a normal biological ontogeny; they must have an origin, a period of growth, and lastly, a period of senescence. During growth a series of integrated biochemical syntheses must produce the basic membranous structure as well as the enzymes involved in oxidative phosphorylation. Although biochemical evidence is rapidly accumulating as to the constitutive processes of protein, lipide, and nucleic acid synthesis, there is no description of how these syntheses are controlled and integrated to produce a functional organelle such as the mitochondrion.

The experiments of Lund, et al (12) with sections of corn root of differing mean cell maturity suggest that a significant growth of mitochondria, including a synthesis of oxidative enzymes, occurs during the expansive phase of cell growth. Plant cell expansion is classically known to be controlled by the native growth hormone, or auxin, indoleacetic acid. Although auxins are defined in terms of their capacity to induce cell expansion (24) they are known to produce manifold effects on cellular metabolism, including increases in respiratory rate (1, 3, 21). Auxins, however, have no promotive effect on the activity of isolated mitochondria (3, 17, 23).

Switzer (23) has demonstrated that mitochondria isolated from soybean seedlings sprayed with the synthetic auxin and herbicide 2,4-D[3] exhibit increased oxidative and phosphorylative activity. He concluded that 2,4-D had formative effects leading to the isolation of particles that retained more of their original (in situ) activity. It appears, however, that an equal-

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[2] This investigation was supported in part by Grant G-3358, National Science Foundation, and represents a portion of a thesis submitted by J. L. Key for the Ph.D. degree.

[3] The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetic acid; DNP, 2,4-dinitrophenol; RNA, ribonucleic acid; RNase, ribonuclease; AMP, adenosine monophosphate.

ly plausible explanation would be that the auxin had initiated biochemical syntheses leading to mitochondria which were more efficient in oxidative phosphorylation.

In the experiments reported here the effect of 2,4-D on the mitochondria of soybean seedlings has been investigated further. The auxin induced a growth of the mitochondria as indicated by changes in composition, by increased size, and by increased phosphorylative activity.

Materials and Methods

Growth of Seedlings: Soybean seeds (*Glycine max* (L.) Merr., var. Hawkeye) were germinated in the dark at 28° C in vermiculite moistened with tap water. The seed was lightly dusted with Spergon (U.S. Rubber Company) to prevent fungal growth on the seed coats. After 60 hrs, when the cotyledons were about 1 in above the surface, the seedlings were sprayed with 15 ml of 5×10^{-4} M recrystallized potassium 2,4-D per 8.5×13 in tray, and returned to the germinator for 24 hrs. This concentration of 2,4-D was used by Switzer. Agronomically it is herbicidal. Comparable trays of seedlings were left unsprayed for production of normal seedling tissue. These treatments are designated in what follows as "2,4-D" and "control", respectively.

Determination of Mitochondrial Activity and Composition: Procedures for isolating mitochondria and determining their oxidative and phosphorylative activity have been described (9, 10, 12). In brief, the mitochondria were isolated from tissue ground in sucrose-phosphate-EDTA as the particulates sedimenting between $800 \times G$ for 5 min and $20{,}000 \times G$ for 15 min, and were once washed in the grinding medium. (The electronmicrograph of ~~fig 1~~ shows the typical contamination of the washed mitochondria with other membranous material. ~~Figure 2~~ is somewhat biased in that it was selected for a cluster of mitochondria; in general, contamination is about the same as in ~~fig 1~~). The washed mitochondria were suspended in 0.5 M sucrose and added to Warburg vessels with the additives previously described. Oxygen consumption and ortho-phosphate disappearance were generally measured for 20 min after 7 min equilibration. Mitochondrial N was determined by digestion and Nesslerization. Alpha-ketoglutarate was used as substrate unless otherwise indicated. The addition of pyruvate with succinate to give high oxidative activity is discussed elsewhere (10).

Analysis of the mitochondria has also been described (9, 10). In brief, mitochondria isolated and washed as above were extracted in cold 0.2 *N* $HClO_4$ to remove soluble nucleotides, followed by extractions in ethanol-ether-chloroform to remove lipides, and in hot 0.5 *N* $HClO_4$ to remove RNA. The nucleotide content of the extracts was determined from 260 to 290 mμ absorption. Lipide-phosphate was determined by digesting the lipide extract and analyzing for ortho-phosphate.

The procedures for pretreating mitochondria with RNase are reported (9). Details appear in Table VI.

Estimates were made of mitochondrial respiration in situ by referring tissue respiration to mitochondrial content as previously described (12), except that the washed mitochondria were analyzed for total N rather than protein. Analyses were also made for total protein N on ice cold 10% TCA precipitates of the cleared homogenates, and the amount of mitochondrial N calculated as percentage of the total (10). The phosphate buffer in the Warburg vessels was labelled with P^{32}, and after measuring respiration the tissue

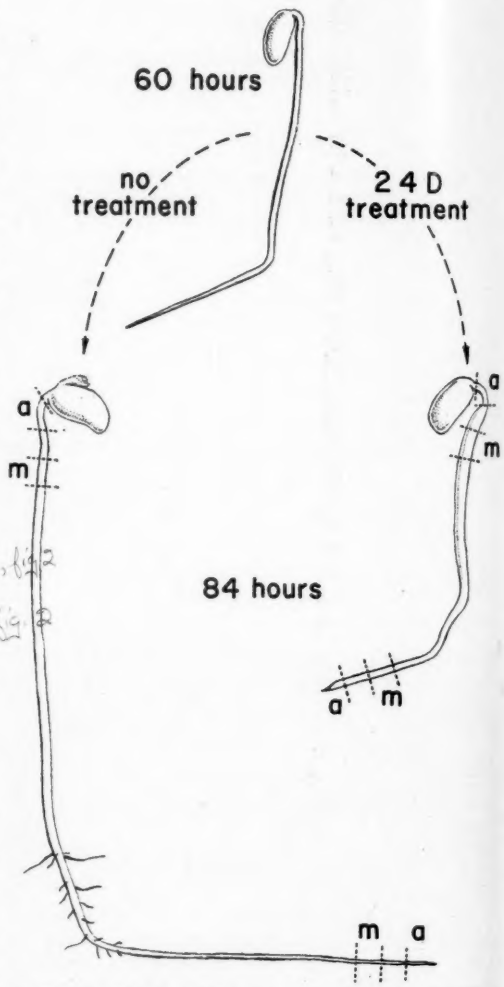

Fig. 1. Morphological effects induced by 2,4-D on soybean seedlings. Apical and maturing tissues used in assessing mitochondrial activity are designated "a" and "m", respectively. These were 0.5 cm sections taken 0.5 cm apart. "Lower hypocotyl" where used in the text refers to tissue from the point of swelling, or potential swelling, to the root.

was removed, thoroughly rinsed with deionized water, digested with nitric acid in porcelain capsules, and the radioactivity determined.

ELECTRON MICROSCOPY: Isolated and once-washed mitochondria were fixed, dehydrated, embedded, sectioned, and viewed as previously described (12).

RESULTS

MORPHOLOGICAL EFFECTS OF 2,4-D: The morphological effects produced by the 2,4-D on soybean seedlings are illustrated in figure 1, and described quantitatively in terms of length and weight in table I. The morphological changes in root and hypocotyl proved to be correlated with changes in mitochondria, and the different tissues selected for study are indicated in figure 1. Detailed anatomical descriptions of comparable changes in beans are given by Beal (2) and Eames (5).

The 2,4-D strongly inhibited the elongation of root and shoot, and the straightening of the hypocotyledonary hook. Normal meristematic activity at the root tip ceased, and the tissue immediately back of the tip swelled. A comparable inhibition occurred in the meristematic and expanding zones above and below the cotyledons. The hypocotyl below the hook exhibited pronounced swelling. Microscopic observation of cross sections of this swollen region showed no initiation of cell division. The swelling is due to cell expansion; the mean diameter of cells in the middle portion of the hypocotyl cortex increased from 77 μ in the control to 123 μ in the 2,4-D treated cortex. Beal (2) reports no cell division in beans until after 48 hrs following 2,4-D application.

The fresh and dry weight data of table I show that hypocotyls of the 2,4-D-treated seedlings were growing, although abnormally.

TABLE I

EFFECT OF 2,4-D TREATMENT ON ELONGATION AND WEIGHT CHANGE OF HYPOCOTYL AND ROOT TISSUE AND WEIGHT CHANGE OF COTYLEDONS FROM 10 SOYBEAN SEEDLINGS

	LENGTH CM/ORGAN	FRESH WEIGHT GM/10 SEEDLINGS	DRY WEIGHT GM/10 SEEDLINGS
Cotyledon			
Initial (60 hrs old)	...	7.87	2.87
Control (84 hrs old)	...	8.12	2.43
2,4-D (84 hrs old)	...	8.83	2.50
Hypocotyl			
Initial	4.15	3.04	0.23
Control	8.00	5.63	0.34
2,4-D	5.40	5.85	0.38
Root			
Initial	4.60	1.74	0.12
Control	8.60	3.23	0.18
2,4-D	5.50	2.45	0.14

For this investigation we distinguished between inhibited and swelling (growing) tissues. The hypocotyledonary hook was taken as representative of tissue with meristematic and elongating cells which were inhibited. The swollen portions of the hypocotyl and root represent tissue with mature or maturing cells which were induced to renew expansive growth, primarily in a lateral direction. The cotyledons represent tissue with limited expansive growth, metabolically active in food mobilization, and not visibly affected by 2,4-D.

EFFECT OF 2,4-D TREATMENT ON RESPIRATORY ACTIVITY OF MITOCHONDRIA: Table II presents representative data on the changes in oxidation and

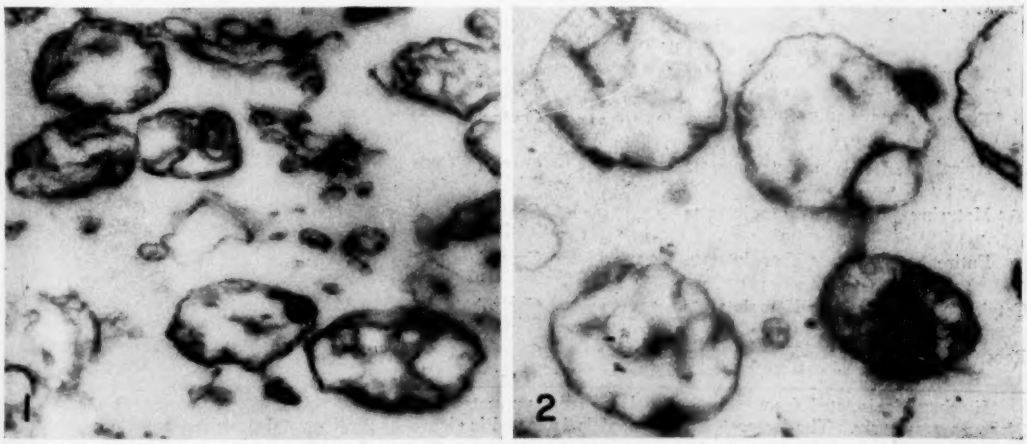

FIG. 2. Representative electromicrographs of mitochondria isolated from soybean hypocotyl. Magnification of both 32,000. 1. Control. 2. 2,4-D-treated 24 hrs previous to isolation. The 2,4-D treatment has induced considerable expansive growth of the mitochondria.

phosphorylation induced by 2,4-D. The results with mitochondria from the entire hypocotyl were from experiments patterned after Switzer's (23) and confirm his report that 2,4-D increases mitochondrial activity. This increase, however, was due entirely to mitochondria from the swollen portion of the hypocotyl. The inhibited apical zone yielded mitochondria lower in activity. The inhibition or promotion of oxidative activity, although invariably found as indicated was not very large compared to the change in phosphorylative activity, particularly with succinate-pyruvate as substrate. Inasmuch as oxidation and phosphorylation were both affected, the P/N ratio provides the best estimate of the total change in production of energy-rich phosphate bonds.

Of basic interest here is the increase in activity shown to accompany normal growth. In normal growth the cells of the apical zone expand, differentiate, and mature, coming in time to a stage comparable to the cells of the maturing zone. Comparing the mitochondria of apical and maturing regions of control tissue, then, provides information on mitochondrial ontogeny. Clearly, there was a sharp increase in P/N accompanying normal growth, presumably induced by endogenous growth hormones. Similar findings have been made with corn root (12) and mesocotyl (J. L. Key, unpublished data).

The mitochondria from the cotyledons were not greatly affected by the 2,4-D. In the roots, which were swollen to within 1 mm of the tip, 2,4-D induced the formation of more active mitochondria.

It was of importance to try to establish whether the activities exhibited by the isolated mitochondria obtained in situ. Estimates of mitochondrial respiration were made by determining the respiration of tissue from 2,4-D-sprayed and control plants, and by referring these rates to the amount of mitochondrial N the tissue contained. Phosphorus accumulation by the tissue was assumed to be proportional to the oxidative phosphorylation occurring within the tissue; the Pacc/O ratio is thought to reflect the phosphorylative efficiency. The basis for this assumption is the conclusion of Hagen et al (8) that orthophosphate uptake by roots is coupled to oxidative phosphorylation. The assumption is not entirely satisfactory, but it provides the only ready basis for estimating oxidative phosphorylation with intact tissue. Although the values are not reported, DNP treatments were included in the tissue experiments to make certain that phosphate accumulation could be uncoupled. With all tissues 10^{-4} M DNP increased the respiration by 15 to 20 % and decreased the phosphate accumulation by 25 to 50 %.

The estimates of in situ activity obtained (table III) agree qualitatively with the oxidative and phosphorylative capacities shown by isolated mitochondria (table II). A comparison of apical and maturing hypocotyl tissues from control plants shows the estimated respiratory and phosphorylative activities of the mitochondria to increase with normal growth. The effect of 2,4-D on maturing hypocotyl tissue was to increase the phosphorylative activity, and on apical tissue to inhibit activity. No significant change in the estimated respiratory rate of the in situ mitochondria occurred as a result of 2,4-D treatment.

Table III also reports mitochondrial N as percentage of total protein N in the cleared homogenates. Both endogenous and 2,4-D-induced growth increase

TABLE II

EFFECT OF 2,4-D SPRAYS ON OXIDATIVE AND PHOSPHORYLATIVE ACTIVITY OF MITOCHONDRIA SUBSEQUENTLY ISOLATED FROM SOYBEAN TISSUE

TISSUE	SUBSTRATE	TREATMENT	$Qo_2(N)$*	P/O**	P/N***
Hypocotyl					
Apical	Succinate-pyruvate	Control	1,505	1.12	150
		2,4-D	1,440	0.59	76
Maturing	Succinate-pyruvate	Control	1,560	1.61	224
		2,4-D	1,620	2.29	331
Entire	Succinate-pyruvate	Control	1,583	1.59	220
		2,4-D	1,644	1.79	267
Apical	*a*-Ketoglutarate	Control	345	1.35	42
		2,4-D	255	0.55	13
Maturing	*a*-Ketoglutarate	Control	740	1.60	105
		2,4-D	794	2.60	184
Entire	*a*-Ketoglutarate	Control	741	1.78	122
		2,4-D	904	2.21	176
Cotyledon	*a*-Ketoglutarate	Control	358	1.51	48
		2,4-D	378	1.30	44
Root	*a*-Ketoglutarate	Control	410	0.98	36
		2,4-D	530	1.40	66

Mitochondria isolated from comparable tissue from untreated seedlings and from seedlings sprayed with 2,4-D 24 hours previously. The hypocotyl tissue used is indicated in figure 1. Entire cotyledons and roots were used.

* μl O_2/hr/mg N.

** μmoles P esterified/μ atom O_2 consumed.

*** μmoles P esterified/hr/mg N.

TABLE III

RESPIRATION AND ORTHOPHOSPHATE UPTAKE BY SOYBEAN TISSUE

TISSUE	SPRAY TREATMENT	MITO N††	Qo_2 (FW)*	Qo_2 (N)**	PACC***	PACC/O† $\times 10^3$
		%				
Hypocotyl Apical	Control	10.9	475	1,533	0.153	3.60
	2,4-D	11.3	535	1,553	0.101	2.12
Maturing	Control	13.3	388	2,300	0.145	4.16
	2,4-D	19.1	319	2,310	0.163	5.72
Root Apical	Control	...	1,410	...	0.50	3.97
	2,4-D	...	548	...	0.31	6.33
Maturing	Control	...	469	...	0.34	8.14
	2,4-D	...	374	...	0.37	11.10

* μl O_2/hr/gm fresh weight.

** μl O_2/hr/mg mitochondrial N. This value was obtained by referring the tissue respiration to the mitochondrial N in the tissue.

*** μmoles PO_4 accumulated/hr/gm fresh weight from 0.002 M potassium phosphate in the Warburg vessel.

† μmoles PO_4 accumulated/μ atom O_2. This value is used as an estimate of the relative phosphorylative efficiency of the mitochondria in situ.

†† Mitochondrial N as percent of total protein N. Mitochondria were washed once in the sucrose-phosphate-EDTA grinding medium.

the relative amount of N sedimenting as mitochondria. This phenomenon has been reported for endogenous growth in corn roots (12).

Only the relative phosphorylative efficiencies were estimated with root tissue. The more mature tissue had Pacc/O ratio double that of the apical tissue. In both apical and maturing tissue the 2,4-D induced a higher Pacc/O. The apical sections of the root used differ from those of the shoot in having tissue swelling (fig 1); the increase in phosphorylative efficiency which accompanies swelling might be expected.

The immediate effects of 2,4-D on the respiration and phosphate uptake by apical and maturing sections of control soybean hypocotyl are shown in table IV. One interesting point illustrated by these data is that the apical section shows a lag in its respiratory response to 2,4-D while the maturing section shows a rather large immediate increase in respiration. The efficiency of phosphate uptake was little affected. It appears that the immediate effect of 2,4-D on in situ mitochondria is to be distinguished from the effects which accompany growth or the inhibition of growth (cf. table III).

EFFECT OF 2,4-D TREATMENT ON MITOCHONDRIAL MORPHOLOGY: Electron microscopy was used to determine if any morphological changes in mitochondria accompanied the increase in activity induced by 2,4-D. Figure 2 shows representative profiles of mitochondrial pellets from 2,4-D and control hypocotyl tissue. The 2,4-D treatment produced swollen mitochondria with less evident internal structure. Swelling apparently reduced the invaginations of the inner membrane thought to produce the cristae (16). "Swelling" is used here as a purely descriptive term to denote the change in size and shape. The swelling appears to result from growth, not from a simple osmotic entry of water, in that it is characterized by increased activity of the mitochondria (table II). The correlation, of course, may be fortuitous. Some comparative observations were made on mitochondria in fixed tissue; the in situ mitochondria also show swelling induced by 2,4-D. No significant changes in mitochondrial morphology were noted to result from 2,4-D in the apical region.

TABLE IV

EFFECT OF 2,4-D ON RESPIRATION AND PHOSPHATE UPTAKE BY SECTIONS OF SOYBEAN HYPOCOTYL

HYPOCOTYL SECTION	2,4-D CONC	Qo_2 (FW)		PACC/O
	mg/l	0–30 Min	30–60 Min	$\times 10^3$
Apical	0	511	513	1.68
	2	499	569	1.77
	10	508	550	1.85
	50	524	574	1.86
Maturing	0	297	310	4.78
	2	351	365	4.85
	10	360	361	4.83
	50	394	366	4.46

The 2,4-D was added to 0.001 M P^{32}-labeled potassium phosphate (pH 6.0) in the Warburg vessel. Oxygen consumption determined after 0.5 and 1 hr, P^{32} content at 1.2 hrs. Mean of duplicate determinations. Apical and maturing sections were cut from control tissue.

EFFECT OF 2,4-D TREATMENT ON MITOCHONDRIAL COMPOSITION: Analyses of the mitochondria from the entire lower hypocotyl and the apical hook were carried out as part of a number of experiments (table V). Two general conclusions can be drawn from the data: normal cell growth and maturation are ac-

TABLE V

ANALYSIS OF MITOCHONDRIA ISOLATED FROM CONTROL AND 2,4-D TREATED SOYBEAN HYPOCOTYLS

TISSUE	TREATMENT	PER MG MITOCHONDRIA N		
		SOL. NUCLEOTIDE μG	RNA MG	LIPIDE-P μMOLES
Apical hook*	Control	10	0.36	1.73
	2,4-D	14	0.36	1.55
Lower**	Control	48	0.39	3.76
	2,4-D	60	0.47	4.64
Standard error of difference		± 2.7	± .07	± 0.15

* 2 experiments

** 6 experiments. Standard error refers to these experiments.

TABLE VI

EFFECT OF RNASE AND DNP ON OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA ISOLATED FROM LOWER HYPOCOTYLS OF CONTROL AND 2,4-D-TREATED SOYBEAN SEEDLINGS

MITOCHONDRIA	$Qo_2(N)$	P/O	P/N
Control	687	1.44	88
+DNP in vessel	712	1.32	84
+RNase pretreatment	514	0.58	27
2,4-D	861	2.34	180
+DNP in vessel	788	2.04	144
+RNase pretreatment	539	0.82	39

Isolated mitochondria were suspended in the sucrose-phosphate-EDTA grinding medium and incubated for 15 min at 28° C with and without 0.9 mg/ml pancreatic RNase (Sigma Chemical Company). After reisolation the mitochondria were added to the Warburg vessels with 10^{-4} M 2,4-dinitrophenol as indicated. Mean values from three experiments.

companied by marked increases in acid soluble nucleotides and lipide phosphate in the mitochondria; 2,4-D-induced growth in the lower hypocotyl also increases these constituents. There was a tendency for the RNA/N ratio to increase with 2,4-D-induced growth, but the change was not statistically significant. Contamination of the mitochondria with microsomal RNA, however, might obscure any increases which were solely mitochondrial.

The dependency of 2,4-D-induced oxidative phosphorylation upon RNA is illustrated in table VI. Pretreatment of mitochondria with RNase will lower the RNA content and uncouple phosphorylation (9). Comparable pretreatment of the mitochondria from the lower hypocotyl with RNase almost entirely erased the gain in total phosphorylative efficiency (P/N) induced by 2,4-D; this was not true of DNP. Furthermore, RNase brought the respiration rates to the same level, while DNP increased the respiration of control mitochondria and decreased that of 2,4-D mitochondria. One inference here is that 2,4-D induced changes in the mitochondrial RNA responsible for or essential to oxidative phosphorylation, but which did not show in the RNA analyses of the mitochondrial pellet.

Table VII shows the dependency of the mitochondrial activity on exogenous AMP. The omission of AMP from the Warburg vessel lowered the activities except for oxidation by mitochondria from 2,4-D-treated tissue. Evidently, the additional soluble nucleotide resulting from 2,4-D-induced growth was sufficient to maintain a high level of oxidation but not of phosphorylation, which required exogenous AMP for maximum activity.

DISCUSSION

The evidence gained from these experiments indicates that 2,4-D can induce a growth of mitochondria in plant cells. This growth is manifested by increased size, by increased mitochondrial N as percentage of total protein N, by increased oxidative and phosphorylative activities, and by increased phospholipide and nucleotides. Growth of mitochondria, however, is confined to tissue which is growing (although somewhat abnormally) as a result of 2,4-D application. Tissues which are inhibited in growth contain mitochondria of lowered activity. The action of 2,4-D, therefore, is not limited to the mitochondria; these simply manifest one aspect of the control of growth by auxin. The normal growth of cells in the soybean hypocotyl (tables II and III) and corn root (12) is also accompanied by increased mitochondrial activity.

The role of 2,4-D (and of endogenous auxin controlling normal growth) is not clear, but for future investigation it would seem profitable to study the relationship between auxin and nucleotide metabolism. The experiments of tables VI and VII indicate that cold-acid soluble nucleotides and RNA are implicated in the increased oxidative and phosphorylative efficiency. Auxins are known to increase the RNA content of tissues (18, 19); Skoog (22) has suggested that they function through polynucleotide metabolism. Of course, there is a very large increase in mitochondrial phospholipide accompanying growth, (table V), but this synthesis could be regulated in turn through the level of cytidine and adenosine phosphates required for synthesis (11).

The growth of mitochondria might explain the increases in tissue respiration which follow auxin application after a lag period (7, 20). There are observations of respiratory increases in isolated tissue (e.g. table IV), however, which occur shortly after application, much in the fashion of those induced by DNP, and before any appreciable growth occurs (6). Indeed, root respiration will increase with auxin concentrations inhibitory to growth and salt uptake (15). These increases have been explained by postulating that auxins cause a rapid utilization of ATP in endergonic growth processes, increasing the ADP and, in turn, the respiration rate (4, 6). The analyses of Marrè and Forti (14) failed to disclose an ADP/ATP ratio favorable to this hypothesis, however. In recent experiments we have confirmed in 2,4-D-sprayed soybeans the increase in ATP noted by Marrè and Forti, and find, in addition, increases in other nucleotides. Perhaps auxin initiates the burst of respiration either

TABLE VII

DEPENDENCY OF MITOCHONDRIA ISOLATED FROM CONTROL AND 2,4-D-TREATED SOYBEAN SEEDLINGS ON EXOGENOUS AMP

TISSUE	AMP	$Qo_2(N)$	P/O	P/N
Control	+	641	2.52	144
	−	560	2.24	112
2,4-D	+	715	2.75	176
	−	703	2.21	139

AMP was omitted from the Warburg vessels as indicated. Mitochondria were isolated from lower hypcotyls. Mean values from 3 experiments.

by simply making more total adenosine phosphates available, or by activating the respiratory enzyme system through its effect on the reduction of glutathione and ascorbic acid as suggested by Marrè and Arrigoni (13).

Summary

Soybean seedlings were sprayed with 2,4-dichlorophenoxyacetic acid; the respiratory activity of the mitochondria from swelling and inhibited tissues was compared with comparable untreated tissue. Tissue swelling was accompanied by increases in phosphorylation and oxidation. Inhibited tissues contained inhibited mitochondria. Studies of tissue respiration and phosphate accumulation indicated that the mitochondrial changes obtain in situ.

Electron micrographs showed the active mitochondria from swelling tissue to be larger and swollen in appearance. During growth these mitochondria increased in acid-soluble nucleotides, phospholipides, and possibly in ribonucleic acid. The increased phosphorylation could be almost completely uncoupled with ribonuclease, and exogenous adenosine monophosphate was not required for maximum substrate oxidation.

Normal growth of soybean tissue involved an increase in mitochondrial activity.

It is concluded that the growth induced by auxins, synthetic or natural, involves a growth of the mitochondria. It is postulated that this growth is regulated through nucleotide metabolism.

Acknowledgement

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Literature Cited

1. Avery, G. S., Jr. 1951. Stimulation of respiration in relation to growth. In: Plant Growth Substances, F. Skoog, ed. Pp. 105–109. University of Wisconsin Press, Madison.
2. Beal, J. M. 1945. Histological reactions of beans to phenoxy compounds. Bot. Gaz. 107: 200–217.
3. Bonner, J. and R. S. Bandurski 1952. Studies of the physiology, pharmacology, and biochemistry of the auxins. Ann. Rev. Plant Physiol. 3: 59–86.
4. Bonner, J., R. S. Bandurski, and A. Millerd 1953. Linkage of respiration to auxin-induced water uptake. Physiol. Plantarum 6: 511–522.
5. Eames, A. J. 1949. Histological effects of treatments with growth regulator substances of the 2,4-D group. Science. 110: 235–236.
6. French, R. C. and H. Beevers 1953. Respiratory and growth responses induced by growth regulators and allied compounds. Amer. Jour. Bot. 40: 660–66.
7. Hackett, D. P. and K. V. Thimann 1953. The nature of auxin-induced water uptake by potato tissue. II. The relation between respiration and water absorption. Amer. Jour. Bot. 40: 183–188.
8. Hagen, C. E., J. E. Leggett and P. C. Jackson 1957. The sites of orthophosphate uptake by barley roots. Proc. Nat. Acad. Sci. 43: 496–506.
9. Hanson, J. B. 1959. The effect of ribonuclease on oxidative phosphorylation by mitochondria. Jour. Biol. Chem. 234: 1303–1306.
10. Hanson, J. B., A. E. Vatter, M. E. Fisher and R. F. Bils 1959. The development of mitochondria in the scutellum of germinating corn. Agron. Jour. 51: 295–301.
11. Kennedy, E. P. 1957. Metabolism of lipides. Ann. Rev. Biochem. 26: 119–148.
12. Lund, H. A., A. E. Vatter and J. B. Hanson 1958. Biochemical and cytological changes accompanying growth and differentiation in the roots of *Zea mays*. Jour. Biophys. Biochem. Cytology 4: 87–98.
13. Marre, E. and O. Arrigoni 1957. Metabolic reactions to auxin. I. The effects of auxin on glutathione and the effects of glutathione on the growth of isolated plant parts. Physiol. Plantarum 10: 289–301.
14. Marre, E. and G. Forti 1958. Metabolic responses to auxin. III. The effects of auxin on ATP level as related to the auxin induced respiration increase. Physiol. Plantarum 11: 36–47.
15. Nance, J. F. 1949. Inhibition of salt accumulation in excised wheat roots by 2,4-Dichlorophenoxyacetic acid. Science. 109: 174–176.
16. Palade, G. E. 1953. An electron microscope study of the mitochondrial structure. Jour. Histochem. Cytochem. 1: 188.
17. Poljakoff-Mayber, A. 1955. The effect of IAA on the oxidative activity of mung bean mitochondria. Jour. Exp. Bot. 6: 321–326.
18. Rebstock, T. L., C. L. Hammer and H. M. Sell 1954. The influence of 2,4-dichlorophenoxyacetic acid on the phosphorous metabolism of cranberry bean plants (*Phaseolus vulgaris*). Plant Physiol. 29: 490–491.
19. Silberger, J. and F. Skoog 1953. Changes induced by indoleacetic acid in nucleic acid contents of tobacco pith tissue. Science 118: 443–444.
20. Smith, F. G. 1948. The effect of 2,4-dichlorophenoxyacetic acid on the respiratory metabolism of bean stem tissue. Plant Physiol. 23: 70–83.
21. Smith, F. G. 1951. Respiratory changes in relation to toxicity. In: Plant Growth Substances, F. Skoog, ed. Pp. 111–120. University of Wisconsin Press, Madison
22. Skoog, F. 1954. Substances involved in normal growth and differentiation of plants. Brookhaven Symposia No. 6 (BNL 258 C-19). 1–21.
23. Switzer, C. M. 1957. Effects of herbicides and related chemicals on oxidation and phosphorylation by isolated soybean mitochondria. Plant Physiol. 32: 42–44.
24. Van Overbeek, J., R. M. Muir, F. W. Went and H. B. Tukey 1954. Nomenclature of chemical plant regulators. Plant Physiol. 29: 307–308.

INHIBITORY EFFECT OF WATER ON OXYGEN CONSUMPTION BY PLANT MATERIALS [1, 2, 3]

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As early as 1891 Devaux (4) showed that wetting the surface of potato tubers markedly decreased the diffusion rate of oxygen through the tissue. If the oxygen uptake rate was sufficiently high, wetting significantly decreased the rate of oxygen consumption.

Ruhland and Ramshorn (10), Kandler (8), James (7), and Allerup (1) have subsequently observed the same or similar effects of water on respiration of various plant tissues. The significance of the water inhibition, however, has apparently drawn little attention, since most of the data on respiration in current literature were obtained with tissues either immersed in or floating on aqueous solutions.

In this report some characteristics of the inhibitory effect of water on oxygen consumption of corn scutellum and other plant tissues are described and their significance is discussed.

MATERIALS AND METHODS

Freshly harvested maturing soybeans (*Glycine max* (L.) Merr., var. Roanoke); germinating soybeans (var. Clark); corn (*Zea mays* L., single-cross hybrid WF9 × M14); barley (*Hordeum vulgare* L., var. Black Hull-less); oats (*Avena sativa* L., var. California Red); potato tubers (*Solanum tuberosum* L.), and carrot roots (*Daucus carota* L., var. sativa, DC.) were used. Seeds were germinated and allowed to grow four to seven days in the dark at 22° C, after which various tissues were excised for use.

Tissues were prepared as follows: individual scutella were cut into four longitudinal pieces, sliced to thicknesses of 0.25, 0.50, and 1.00 mm, or used intact. All pieces in a group were pooled, and eight to ten pieces (about 100 mg, fresh weight) were used

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TABLE I

EFFECT OF WATER ON OXYGEN UPTAKE OF VARIOUS PLANT TISSUES

MATERIALS	OXYGEN UPTAKE (μl O_2/HR/G)		
	WITHOUT WATER AIR	WITH WATER AIR	WITH WATER O_2
Immature soybean seeds			
Intact	340	120	370
2 mm Slice	625	250	625
Germinating soybeans (4 *days*)			
Intact cotyledon	900	600	850
Root	460	330	470
Hypocotyl	400	385	430
Cotyledon*	127	113	190
Germinating corn			
Whole scutellum	1,900	680	2,000
Shoot	1,000	630	930
Root	570	360	580
Corn coleoptile	290	250	310
Barley coleoptile	280	160	255
Oat coleoptile	420	350	375
Carrot root	190	160	170
Carrot root previously washed with water	115	140	160
Potato tubers	51	41	46

* Seeds were soaked in water for 14 hrs at 4° C.

for each measurement. Immature soybean seeds of about 75 mg dry weight (about half the total dry weight at maturity) were used intact immediately after harvesting or were sliced to 2 mm thickness; intact seeds were also used after a storage period of one month at 4° C. Roots, hypocotyls, shoots, and coleoptiles were cut to 3 mm lengths, pooled, and weighed into the vessels. Carrot and potato tissues were cut to discs 7 mm in diameter by 1 mm thick. A quantity of tissue sufficient to absorb more than 100 µl of oxygen per hour was used in each vessel except that in experiments with potato slices 500 mg were used. Materials were not washed. Oxygen uptake measurements were started as rapidly as possible.

Respiratory gas exchange was measured in 15 ml Warburg flasks. Temperature was 29° C except as noted otherwise. Vessels were shaken horizontally with an amplitude of about 4 cm at a rate of 115 strokes per minute. For oxygen measurements, the center well contained 2 × 2 cm pieces of filter paper wetted with 0.2 ml of 4 *N* KOH. Carbon dioxide output was measured by the indirect method (12), correcting for retention of CO_2 as follows: two vessels without KOH each contained 0.5 ml of 5 % trichloracetic acid in the side arms; the acid of one vessel was tipped into the main chamber at the start of the measurement and that of the other at the end. A third vessel containing KOH was used for measurement of oxygen uptake. Except as stated otherwise, 2.0 ml of water was used in the main chamber for experiments with water. For without water experiments, 0.3 ml of water was placed in the side arm to maintain high humidity. The gas phase was air except when replaced with oxygen as indicated. The oxygen environment was established by flushing each vessel with 0.5 l tank oxygen. Since the duration of respiration measurements was less than 60 min, influence of contaminating bacteria was considered negligible.

Results and Discussion

The results of survey experiments on the inhibitory effect of water on tissues of several species are summarized in table I. A decrease of oxygen uptake upon adding water was of common occurrence. The magnitude of the inhibition differed between plant species and also between tissues of a given species. In all cases, however, the decreased activity due to addition of water was fully or partially recovered upon replacing air with oxygen.

Because of the magnitude of water effect on corn scutellum, this tissue was selected for subsequent experiments.

Table II

Effect of Thickness of Corn Scutellum on Oxygen Uptake*

Thickness (mm)	Oxygen uptake (ml O_2/hr/g)			
	With water		Without water	
	Air	Oxygen	Air	Oxygen
0.25	1.22	1.38	1.20	1.26
0.50	1.26	1.98	1.86	1.82
1.00	1.06	1.88	1.90	1.78
Quartered longitudinally	0.98	1.81	1.98	1.86

* Scutella from 6-day-old corn seedlings were used.

The effect of tissue thickness on water inhibition is shown by the data of table II. As thickness was increased, so was the magnitude of the inhibition. Except for the 0.25 mm slices, oxygen uptake in the absence of water was virtually independent of tissue thickness. Substitution of oxygen for air in the gas phase did not accelerate oxygen uptake in the absence of water at any tissue thickness. This indicated that in the absence of water oxygen, uptake was not limited by oxygen supply to respiratory sites.

Respiration of 0.25 mm slices was always less than that of thicker material. Evidently it is limited by some factor other than oxygen diffusion. For instance, in slices of this thickness there would be a greater ratio of damaged to intact cells than in thicker slices.

The temperature coefficient of oxygen consumption over the range 15 to 36° C is shown in table III.

Table III

Temperature Coefficient of Oxygen Uptake by Corn Scutella*

Temperature °C	With water				Without water			
	Air		O_2		Air		O_2	
	O_2 Uptake	Q_{10}	O_2 Uptake	Q_{10}	O_2 Uptake	Q_{10}	O_2 Uptake	Q_{10}
15	0.60		0.76		0.68		0.70	
		1.52		2.30		2.57		2.44
22	0.80		1.36		1.32		1.30	
		1.25		1.86		1.84		1.65
29	0.94		2.09		2.02		1.85	
		1.29		1.68		1.62		1.80
36	1.12		3.00		2.82		2.80	

* 5-day-old corn scutella, quartered longitudinally. Oxygen uptake is expressed as ml/hr/g fresh weight.

At 15° C the oxygen uptake rates under all four conditions were similar. As the temperature was increased, the rate in the presence of water in air increased more slowly than those under the other three conditions. Thus, in water at 29° C the rate was about half that in air (0.94 vs 2.02). The temperature coefficient of oxygen uptake of scutellum tissue in the presence of water in air (1.3 to 1.5, table III) is similar to that of oxygen diffusion in water (7), and is less than those typical of enzymatically catalyzed reactions (6). Thus, in the presence of water in air, the rate-limiting process in oxygen uptake is probably the diffusion of oxygen. Under the other three experimental conditions of table III, temperature coefficients were sufficiently high to indicate that the rate-determining steps were enzymatically catalyzed reactions.

The results presented in tables I, II, and III suggest that water inhibition is caused by a decrease in the oxygen diffusion rate. This may be due to the water surrounding the scutellum tissue or to internal water. The shaking rate was sufficient to provide oxygen up to 400 µl/hr without restriction due to the diffusion of oxygen through the external water (12). It is improbable, therefore, that the inhibition is due to external water, and the results shown in table IV exclude this possibility. The addition of even minute amounts of water decreased the oxygen uptake markedly. Nearly 50 % inhibition was obtained from addition to each vessel of 0.3 ml of water, an amount far less than that required to cover the tissue. The decreased rate of oxygen diffusion is therefore attributed mainly to conditions within the tissue itself. When scutellum tissue was soaked in water and then blotted dry, the rate of oxygen uptake was diminished.

TABLE IV

EFFECT OF ADDITION OF WATER AND PREVIOUS SOAKING ON RESPIRATION OF CORN SCUTELLA*

TREATMENT	OXYGEN UPTAKE-%
No water added	98 %
0.1 ml Water added	68
0.3 ml Water added	56
0.6 ml Water added	54
1.3 ml Water added	40
3.0 ml Water added	37
10 Min soaking period	80
30 Min soaking period	84
180 Min soaking period	65

* Each Warburg vessel contained about 100 mg of 4-day-old corn scutella quartered longitudinally. Oxygen uptake in the absence of water in air, previously measured for 30 minutes, was 2.26 ± 0.14 ml O_2/hr/g. This value was taken as 100 %. Subsequently, water indicated in the table was added and oxygen uptake was again measured. Scutella in other flasks were removed and soaked in 50 ml of distilled water per 100 mg of tissue for indicated periods. Oxygen uptake in the absence of water in air was measured again after blotting to remove surface water. Because of the gradual increase of rate with time, readings for only the first 30 minutes were taken.

TABLE V

EFFECT OF OXYGEN SUPPLY ON RESPIRATORY QUOTIENT OF CORN SCUTELLUM

EXPT NO.		WITH WATER		WITHOUT WATER	
		AIR	O_2	AIR	O_2
1*	O_2 uptake (ml/hr/g)	1.04	1.91	1.99	1.98
	CO_2 output (ml/hr/g)	0.97	1.24	1.18	1.00
	Respiratory quotient	0.93	0.65	0.65	0.58
2*	O_2 uptake (ml/hr/g)	1.20	2.23	2.49	2.45
	CO_2 output (ml/hr/g)	1.48	1.51	1.56	1.51
	Respiratory quotient	1.23	0.68	0.63	0.60

* 6- and 5-day-old corn scutella quartered longitudinally were used in expts. 1 and 2, respectively.

However, the activity of such tissue gradually increased to the original level so that even during the first 30 min, there is less inhibition than occurs in the presence of small amounts of water.

Table V shows the marked effect of water addition on the respiratory quotient of scutellum tissue. CO_2 production varied little under the four conditions, in contrast to the decrease in oxygen uptake in the presence of water. Consequently, the respiratory quotient was increased by adding water. This result is similar to those of Ruhland and Ramshorn (10), and Kandler (8), but does not confirm that of Steward, et al (11).

In a preliminary experiment no accelerating effect of 2,4-dinitrophenol (DNP) on the oxygen uptake of intact or sliced corn scutellum in the presence of water in air was observed. The data in table VI, however, indicate that oxygen uptake by slices or longitudinal quarters is accelerated in an oxygen atmosphere by DNP concentrations up to 3.5×10^{-5} M. This result also supports the view that in the presence of water, oxygen diffusion through the tissue is the process determining the rate of oxygen uptake. The fact that greater acceleration was ob-

TABLE VI

EFFECT OF 2,4-DINITROPHENOL (DNP) ON OXYGEN UPTAKE BY CORN SCUTELLUM

DNP FINAL CONCENTRATION	RELATIVE OXYGEN UPTAKE BY SCUTELLA CUT IN		
	LONGITUDINAL QUARTERS	1.00 MM SLICES	0.50 MM SLICES
0	100 %	100 %	100 %
10^{-6} M	104	99	110
3.5×10^{-6} M	...	...	112
10^{-5} M	114	124	129
3.5×10^{-5} M	123	145	142

Each reaction mixture contained 0.03 M KH_2PO_4, pH 5.0. Gas phase was oxygen. Scutella were from 6- and 7-day-old seedlings. Data are expressed as the percentages of activities in the absence of DNP.

tained with 1.00 and 0.50 mm slices than with longitudinal quarters suggests that, even in an oxygen atmosphere, the oxygen diffusion rate was not sufficient to fulfill the elevated requirement for oxygen in the presence of DNP.

The present study shows that the inhibitory action of added water is caused by a decrease in the oxygen diffusion rate within the tissue itself. The diffusion rate is much higher in the absence of water than in its presence. The presence of an intercellular gas phase in many plant tissues (5) and its contribution to the high oxygen diffusion coefficients of potato tubers (2) and carrot roots (3) has already been demonstrated. It is therefore assumed that water lodged along the diffusion path decreased the oxygen diffusion rate in these tissues. That the water inhibition is not simply a surface phenomenon is indicated by the decrease in inhibition which accompanied a decrease in tissue thickness. If inhibition resulted from a surface film of water, it should be largely independent of tissue thickness.

The magnitude of the water inhibition may also vary with age of the tissue. Results in figure 1 show how the oxygen uptake of soybean cotyledons changes during germination.

In a preliminary experiment it was found that 0.25, 0.50, and 1.00 mm cotyledon slices had similar oxygen uptake rates in water with oxygen as the gas phase. It was therefore assumed that under the conditions of curve II (fig 1) oxygen supply was sufficient to support maximum oxygen uptake. Respiration may have been limited by some other factor such as phosphate acceptor supply (9). The values of curve I were therefore considered to approximate more nearly the real oxygen uptake capacity of the tissue. The optimal concentration of DNP for maximum activation was somewhat different for tissues of different ages. For 1- to 3-day-old tissue, the optimal concentration of DNP was 10^{-4} M, whereas for 4-day-old and older tissue it was 3.5×10^{-5} M. Data in figure 1 were obtained with concentrations giving maximum activation at each age.

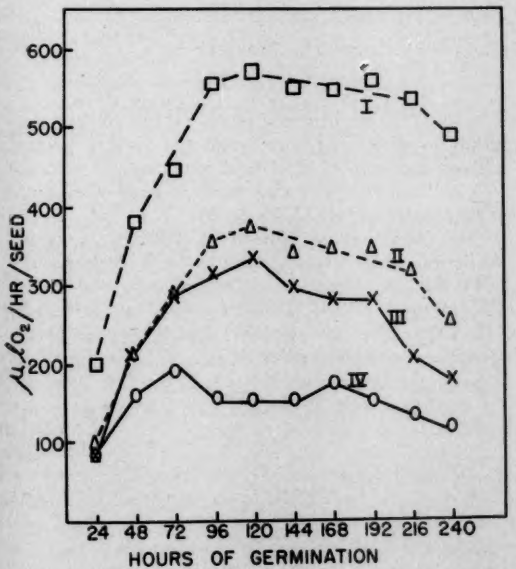

FIG. 1. Oxygen uptake of soybean cotyledons under various conditions. I. 0.50 mm slices in DNP (1st 3 days 10^{-4} M, 4th day and subsequently 3.5×10^{-5} M), gas phase oxygen; II. 0.50 mm slices in water, gas phase oxygen; III. whole cotyledons in absence of water, gas phase air; IV. whole cotyledons in water, gas phase air.

The oxygen uptake of whole cotyledons in air and in the absence of water (curve III) is considered to approximate the actual rate of oxygen uptake of the intact tissue. However, the temperature difference between measurements (29° C) and germination (22° C) and injuries caused by removal of hypocotyls may make observed values somewhat different from the actual ones. Conditions for curve IV were the same as for curve III except for the presence of water. The difference between curve IV and curve II shows the magnitude of diffusion inhibition in the presence of water.

The respiratory capacity of the cotyledon (curve I) increases rapidly following germination and continues at a high level at least through the tenth day. On the other hand, respiration in air and in the absence of DNP and water (curve III) reaches its maximum on the fifth day. It then declines faster than under the conditions of curve I. During the first three days, diffusion through the tissue can supply oxygen just sufficient to fulfill the decreased requirement for oxygen which exists in the absence of DNP (curves II and III vs. curve I). After the third day, diffusion is too slow to fulfill even this requirement. At least 35 % of the respiratory capacity is not in use throughout the whole ten day period (curve I vs. curve III) because of an insufficient supply of phosphate acceptors and the lack of oxygen after the third day.

SUMMARY

Adding water to corn, soybean, and barley tissues resulted in marked decreases of oxygen uptake. Carrot, oat, and potato tissues were affected similarly but to a smaller degree. Substitution of oxygen for air in the gas phase partially or completely reversed the effect of added water. The inhibitory action of water on oxygen uptake of corn scutellum was attributed to the decreased rate of oxygen diffusion through the tissues for the following reasons: I. the inhibition was removed by replacing air with oxygen; II. the inhibitory effect decreased with decreasing thickness down to a tissue thickness of 0.50 mm; III. in the presence of water the uptake of oxygen had a low temperature coefficient similar to that of oxygen diffusion through water; IV. minute amounts of water and previous soakings had inhibitory effects.

The oxygen uptake of intact soybean cotyledons during germination is rate-limited by the lack of

sufficient phosphate acceptors and by the rate of oxygen diffusion.

Literature Cited

1. Allerup, S. 1959. Respiration in water imbibing barley caryopses. Physiol. Plantarum 12: 118–123.
2. Burton, W. G. 1950. Studies on the dormancy and sprouting of potatoes. I. The oxygen content of the potato tuber. New Phytologist 49: 121–134.
3. Briggs, G. E., and R. N. Robertson 1948. Diffusion and absorption in disks of plant tissue. New Phytologist 47: 265–283.
4. Devaux, H. 1891. Etude Experimentale Sur L'Aeration des Tissus Massifs Ann. sci. nat. Bot. XIV. 297–395.
5. Denny, F. E. 1946. Gas content of plant tissue and respiration measurement. Contrib. Boyce Thompson Inst. 14: 257–276.
6. Gortner, R. A., Jr., and W. A. Gortner 1949. Outlines of Biochemistry. John Wiley & Sons, Inc., New York.
7. James, W. O. 1953. Plant Respiration. Clarendon Press, Oxford.
8. Kandler, O. 1950. Untersuchungen über den Zusammenhang zwischen Atmungsstoffwechsel und Wachstumsvorgängen bei in vitro kultivierten Maiswurzeln. Zeitschrift für Naturforschung 5b: 203–211.
9. Laties, G. G. 1957. Respiration and cellular work and regulation of the respiration rate in plants. Survey of Biological Progress, Vol. 3: 215–299. Academic Press, Inc., New York.
10. Ruhland, W., and K. Ramshorn 1938. Aërobe gärung in Aktiven Pflanzlichen Meristemen. Planta 28: 471–514.
11. Steward, F. C., R. Wright, and W. E. Berry 1932. The absorption and accumulation of solutes by living plant cells. III. The respiration of cut discs of potato tubers in air and immersed in water, with observations upon surface: volume effects and salt accumulation. Protoplasma 16: 576–611.
12. Umbreit, W. W., R. H. Burris, and J. F. Stauffer 1957. Manometric Techniques. Burgess, Minneapolis.

PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS IX. PHOTOSYNTHETIC PHOSPHORYLATION AND CO_2 ASSIMILATION IN DIFFERENT SPECIES[1]

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Understanding the role of chloroplasts in photosynthesis has been greatly increased during the past five years by experiments with chloroplasts isolated from the leaves of one species, spinach (*Spinacia oleracea* Linn.). Previously, the only experimentally documented photochemical activity of chloroplasts isolated from several species, including spinach, was the Hill reaction (18) in which illuminated chloroplasts evolve oxygen in accordance with equation I, where

$$A + H_2O \xrightarrow{\text{Light}} AH_2 + 1/2\ O_2 \qquad \text{I}$$

A represents a non-physiological electron or hydrogen acceptor such as ferricyanide or benzoquinone (27).

The new work with spinach chloroplasts has provided direct experimental evidence for the capacity of isolated chloroplasts to assimilate CO_2 photosynthetically to the level of starch and sugars (4, 5, 1, 17, 25). Previously, the view that photosynthetic CO_2 assimilation in green plants is, like oxygen evolution, localized in chloroplasts, was at first asserted without the support of critical experimental evidence (22, 23) and was later abandoned because of evidence to the contrary (18, 15, 14, 21, 6).

By fractionating spinach chloroplasts, CO_2 assimilation proper was shown to be a dark process (24), but one dependent on assimilatory power, i.e., $TPNH_2$ and ATP[4], formed by two light reactions (11, 13), cyclic (equation II) and non-cyclic photophosphorylation (equation III).

$$n\ ADP + n\ H_3PO_4 \xrightarrow{\text{Light}} n\ ATP \qquad \text{II}$$

$$2TPN + 2H_2O + 2ADP + 2H_3PO_4 \xrightarrow{\text{Light}} 2TPNH_2 + O_2 + 2ATP \qquad \text{III}$$

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[4] The following abbreviations will be used: TPN, $TPNH_2$, oxidized and reduced forms of triphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P, *ortho*phosphate; FMN, riboflavin phosphate; Tris, Tris(hydroxymethyl)-aminomethane buffer, neutralized with HCl.

The assimilatory power generated by reactions II and III is used for CO_2 assimilation in accordance with reaction IV.

$$CO_2 + 2TPNH_2 + n\,ATP \longrightarrow (CH_2O) + 2TPN + n\,ADP + n\,H_3PO_4 \quad IV$$

The sum of reactions II, III, and IV gives the overall equation for photosynthesis:

$$CO_2 + 2H_2\overset{*}{O} \xrightarrow{\text{Light}} (CH_2O) + \overset{*}{O}_2 + H_2O \quad V$$

A non-physiological variant of reaction III is reaction VI in which TPN is replaced by ferricyanide

$$4Fe^{3+} + 2H_2O + 2ADP + 2H_3PO_4 \xrightarrow{\text{Light}} 4Fe^{2+} + O_2 + 2ATP + 4H^+ \quad VI$$

(represented here by Fe^{+++}). The Hill reaction (equation I) was shown to be an uncoupled photophosphorylation (11, 13, 19), i.e., that fragment of reaction VI which represents the electron transport when it is not accompanied by ATP formation.

It was desirable to demonstrate the newly found photosynthetic capacity of isolated chloroplasts in other species besides spinach, especially since the work with spinach was used in formulating a concept of photosynthesis (7) no longer based on the photolysis of water as the common denominator of photosynthesis in green plants and photosynthetic bacteria (26). This article presents evidence that chloroplasts isolated from the leaves of sugar beet *Beta vulgaris* Linn. (Chenopodiaceae), tobacco *Nicotiana tabacum*, Linn. (Solanaceae), pokeweed[5] *Phytolacca americana*, Linn. (Phytolaccaceae), sunflower *Helianthus annuus*, Linn. (Compositae) and New Zealand spinach, *Tetragonia expansa* Murr. (Aizoaceae) can carry out the same three processes which were recently discovered in spinach chloroplasts: cyclic photophosphorylation, non-cyclic photophosphorylation and assimilation of CO_2 to the level of carbohydrates.

Preliminary reports of some of these findings have been made previously (6, 31).

Methods

The plants were all grown in the greenhouse in water culture using the nutrient solution technique described elsewhere (3, 8). Mature leaves were used in preparing the chloroplasts. Broken chloroplast particles (P_{1s} or C_{1s}) and chloroplast extract (CE) were made by the methods previously described (32). The only modification was that 0.5 molar NaCl was used instead of 0.35 molar NaCl for grinding the leaves and washing the chloroplasts in the case of sugar beet and tobacco. Chloroplasts from the other species were made in 0.35 molar NaCl. Measurements of chlorophyll (3), phosphorylation (10), TPN reduction (12), and CO_2 fixation (1) were made as described previously.

Results

Cyclic Photophosphorylation: Cyclic photophosphorylation (equation II) is a photochemical reaction of chloroplasts in which all the trapped light energy is converted into ATP. Cyclic photophosphorylation is not accompanied by oxygen evolution (or absorption), and is independent of CO_2 assimilation. No reductant for CO_2 assimilation is formed; the sole product is ATP, which is formed by esterification of inorganic phosphate (cf. review, 6).

Work with spinach chloroplasts has shown that there are two kinds of cyclic photophosphorylation, one catalyzed by FMN[4] and the other by vitamin K compounds (30, 28, 32). The FMN and vitamin K pathways, (or systems) are similar in many respects, but may be distinguished in that the FMN system is more sensitive to inhibition by *o*-phenanthroline and dinitrophenol than the vitamin K system (32). Moreover, the FMN pathway differs from the vitamin K pathway in its requirement for TPN (32) and chloride ions (7) (J. Bové, C. Bové, F. R. Whatley, and D. I. Arnon. 1959. Manuscript in preparation.)

Table I shows the two pathways of cyclic photophosphorylation in isolated chloroplasts from the different species. A dark control is shown in the FMN system, to demonstrate the strict light dependence of the process, which was also observed in experiments with the vitamin K system.

Non-cyclic Photophosphorylation: In non-cyclic photophosphorylation (equation III) ATP formation is coupled with the reduction of TPN and evolution of oxygen; the generation of the energy-rich

Table I

Cyclic Photophosphorylation by Chloroplasts from Different Plants

Plant	P esterified (μmoles/30 min/0.1 mg chl)		
	FMN system		Vit K_3 system
	Light	Dark	Light
Spinach	7.6	0.3	7.9
Sugar beet	8.4	0.2	7.8
Pokeweed	6.6	0.3	7.7
Tobacco	4.3	0.3	5.1
Sunflower	4.2	0.2	3.9
Tetragonia	8.3	...	7.6

The reaction mixture (3 ml final volume) contained, in micromoles: tris[4], pH 8.3, 80; KCl, 20; $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10; Na ascorbate, 10; and chloroplast fragments (C_{1s}), containing 0.1 mg chlorophyll. In addition the FMN system contained, in micromoles: TPN, 0.3; and FMN, 0.1; in the vitamin K_3 system the TPN and FMN were omitted and 0.3 μmole vitamin K_3 was added. The reaction was run under N_2 at 15° C for 30 min and the esterification of phosphate was measured as described previously (10).

[5] Drs. C. S. French and Helen M. Habermann suggested the use of this species and kindly supplied the seed.

TABLE II

NON-CYCLIC PHOTOPHOSPHORYLATION BY CHLOROPLASTS FROM DIFFERENT PLANTS

PLANT	$TPNH_2$ FORMED (μMOLES)	OXYGEN EVOLVED (μATOMS)	P ESTERIFIED (μMOLES)
Spinach	3.3	4.0	6.5
Sugar Beet	3.3	3.4	6.4
Pokeweed	1.8	1.6	3.7
Tobacco	2.6	2.0	4.4
Sunflower	2.7	1.2	3.0

The reaction mixture contained (3 ml final volume), in micromoles: Tris, pH 8.3, 80; KCl, 20; TPN, 4; $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10; sodium ascorbate, 10; chlorophyll-free chloroplast extract from chloroplasts containing 2 mg chlorophyll; and chloroplast fragments (P_{1a}) containing 0.25 mg chlorophyll. Experiments were run in the light at 15° C under nitrogen for 30 min. $TPNH_2$ was measured by its absorption at 340 mμ (12), oxygen was measured manometrically (KOH in center well), and esterification of phosphate was measured as described previously (10).

pyrophosphate bonds of ATP accounts for only a portion of the light energy captured by the chloroplasts. Another portion is used for forming $TPNH_2$. Non-cyclic photophosphorylation provides the three products of the light phase of photosynthesis in green plants: molecular oxygen, and the two components of assimilatory power (11) required for converting CO_2 into sugars, namely, $TPNH_2$ and ATP. Table II illustrates the ability of chloroplasts from different species to carry out non-cyclic photophosphorylation.

The salient features of non-cyclic photophosphorylation are that ATP formation is accompanied by TPN reduction and oxygen evolution. As shown in table II, this was found with all species investigated. The stoichiometric relation, however, expressed by equation III, between the pairs $TPNH_2$ and oxygen, and ATP and $TPNH_2$ was reasonably good for the first pair (except in sunflower) but not for the second. More ATP was formed than $TPNH_2$, suggesting that some cyclic photophosphorylation also occurred in the reaction mixture.

It was previously reported (12) that the P: 2e ratio of one between ATP formed and TPN reduced was observed only under special experimental conditions. In the case of spinach (12) a P: 2e ratio of one was obtained by omitting ascorbate, washing chloroplasts and dialyzing the TPN-reducing factor, supplied by the chloroplast extract (CE). These special experimental conditions, which were considered to be beyond the scope of this investigation, were necessary to eliminate the additional ATP formation by the cyclic photophosphorylation pathway which is operative, albeit at a lower rate, even without added FMN or vitamin K (4, 9). The degree of suppression of this residual cyclic photophosphorylation may vary from species to species and also in different chloroplast preparations of the same species, depending upon the carryover of the cofactors of cyclic photophosphorylation that are present in the intact leaf.

ATP FORMATION COUPLED WITH FERRICYANIDE REDUCTION: A non-physiological variant of Reaction III is Reaction VI, in which TPN is replaced by ferricyanide. Reaction VI, first found with spinach chloroplasts, has led to an interpretation of the Hill reaction as an uncoupled photophosphorylation, i.e., as a measure of photochemical electron transport which is proceeding without an associated phosphorylation reaction (11, 12, 13, 19, 20). This conclusion is based upon the observation that, with spinach chloroplasts, the rate of oxygen evolution in the Hill reaction, when it is coupled with phosphorylation, is higher than in the conventional Hill reaction without phosphorylation (equation I). As shown in table III photophosphorylation coupled with reduction of ferricyanide and oxygen evolution, was observed with chloroplasts of several different species. In general, as in spinach chloroplasts, the rate of oxygen evolution accompanying ferricyanide reduction was increased by coupling the system with photophosphorylation (11, 13, 19).

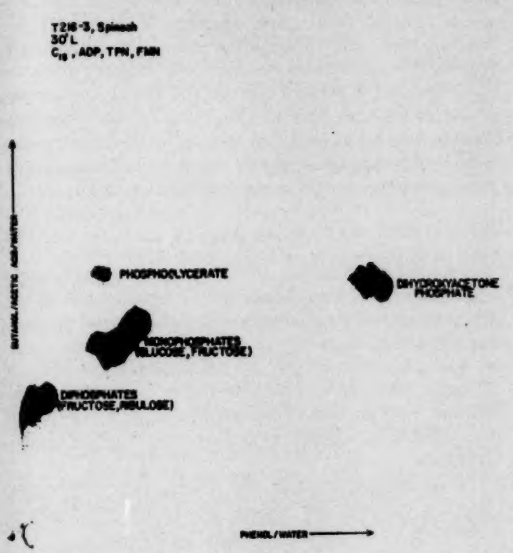
CO_2 ASSIMILATION: Photosynthetic CO_2 assimilation for spinach was first demonstrated with whole chloroplasts (4, 1, 17, 25), and subsequently with broken chloroplasts supplemented with chloroplast extract and a number of cofactors (29). In experiments carried out with other species the ability to

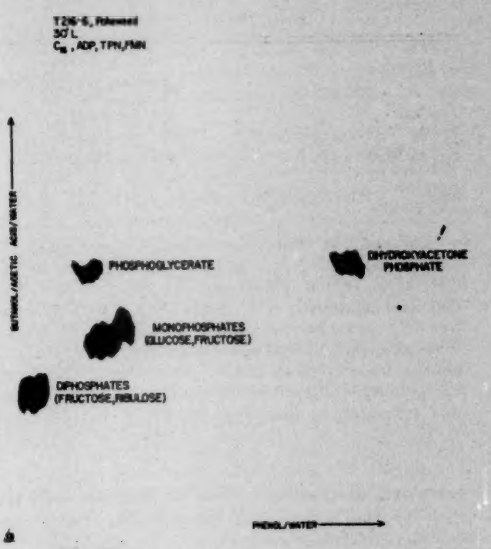
TABLE III

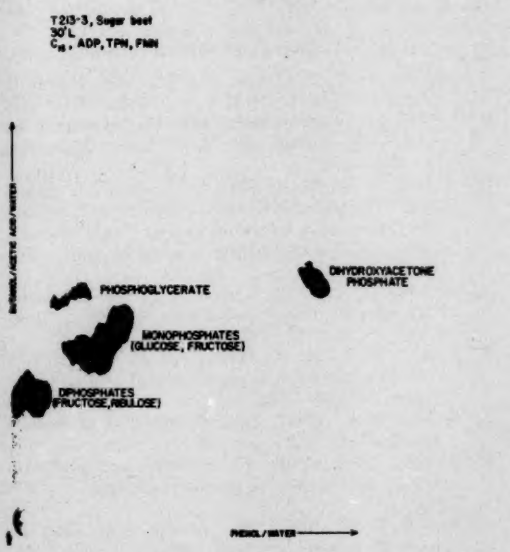
HILL REACTION, WITH AND WITHOUT COUPLED PHOSPHORYLATION, BY CHLOROPLASTS FROM DIFFERENT PLANTS

PLANT	HILL REACTION	HILL REACTION COUPLED WITH PHOSPHORYLATION	
	OXYGEN EVOLVED (μATOMS)	OXYGEN EVOLVED (μATOMS)	P ESTERIFIED (μMOLES)
Spinach	4.8	6.2	5.8
Sugar Beet	5.1	7.5	6.6
Pokeweed	5.8	6.0	4.9
Tobacco	3.8	4.7	1.8
Sunflower	2.4	2.1	1.2
Tetragonia	...	5.2	6.3

The Hill reaction mixture contained (3 ml final volume), in micromoles: tris, pH 8.3, 80; KCl, 20; $K_3Fe(CN)_6$, 15; and chloroplast fragments (P_{1a}) containing 0.1 mg chlorophyll, prepared as described previously (10). The light reaction was carried out at 15° C under nitrogen for 30 min, and the oxygen evolution measured manometrically (with KOH in the center well). For the experiments with coupled phosphorylation the reaction mixture contained, in addition, in micromoles: $MgCl_2$, 5; ADP, 10; $K_2HP^{32}O_4$, 10. The oxygen evolution was measured manometrically and the phosphate esterified as described previously (10).







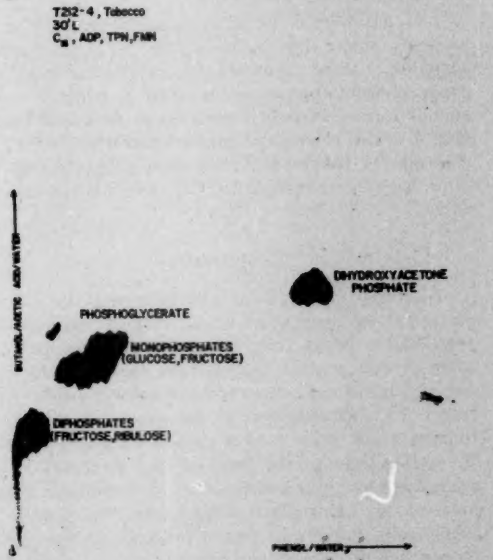


FIG. 1 (*upper left*). Products of CO_2 assimilation by illuminated chloroplasts isolated from spinach. Experimental conditions are described in the legend to table IV.

FIG. 2 (*lower left*). Products of CO_2 assimilation by illuminated chloroplasts isolated from sugar beet. Experimental conditions are described in the legend to table IV.

FIG. 3 (*upper right*). Products of CO_2 assimilation by illuminated chloroplasts isolated from pokeweed. Experimental conditions are described in the legend to table IV.

FIG. 4 (*lower right*). Products of CO_2 assimilation by illuminated chloroplasts isolated from tobacco. Experimental conditions are described in the legend to table IV.

TABLE IV

CO_2 FIXATION BY CHLOROPLASTS FROM DIFFERENT PLANTS

PLANT	CO_2 FIXED (μMOLES/MG CHL/HR)
Spinach	2.2
Sugar Beet	3.5
Pokeweed	2.2
Tobacco	1.7
Sunflower	1.4
Tetragonia	2.0

The reaction mixture contained, in micromoles: Tris, pH 7.4, 80; $MgCl_2$, 5; $MnCl_2$, 2; Na ascorbate, 10; TPN, 0.3; ADP, 0.5; K phosphate, pH 7.4, 5; FMN, 0.0001; glucose-1-phosphate, 0.3; $NaHC^{14}O_3$, 10; chlorophyll-free chloroplast extract made from chloroplasts containing 2 mg chlorophyll; and chloroplast fragments (C_{1a}) containing 0.5 mg chlorophyll. The reaction was run at 15° C under N_2 for 30 min in the light. The CO_2 fixation was measured as described previously (1).

carry out extracellular CO_2 assimilation was tested with broken chloroplast preparations. The results are summarized in table IV.

CO_2 assimilation in all cases has progressed to the level of sugar phosphate (table IV). Individual compounds were identified by radioautography and paper chromatography (1). The pattern of compounds formed in light from CO_2 is shown in figures 1 to 4. The close similarity of products formed by chloroplasts from different species suggests that the same basic mechanisms for CO_2 assimilation are involved.

DISCUSSION

Although extracellular photosynthesis by chloroplasts cannot be equated in all respects with photosynthesis in intact cells, it retains the principal features of that process, and permits their investigation and isolation from concurrent cellular activities. Extracellular photosynthesis by chloroplasts is similar to that in whole cells in that carbon dioxide reduction by visible light to the level of carbohydrates is accompanied by oxygen evolution. Extracellular photosynthesis by chloroplasts differs from that process in whole cells, notably in that it proceeds independently of concurrent cellular activities (such as respiration, which cannot be divorced from photosynthesis in intact cells (16)).

Separation of photosynthesis by chloroplasts from the structural and functional complexity of the whole cells has opened a way for investigating the central problem of photosynthesis, the conversion of light into chemical energy, in a manner which has already forced a re-examination of certain established concepts in photosynthesis (7). It was therefore deemed especially important to base the new conclusions about the mechanism of photosynthesis on evidence that the three key photosynthetic processes recently identified in spinach chloroplasts, CO_2 assimilation, cyclic, and non-cyclic photophosphorylation, also occur in chloroplasts isolated from other species. The aim of this investigation was to establish this point in principle, leaving for subsequent research the determination for each species of those experimental conditions, which result in highest rates of CO_2 fixation and photosynthetic phosphorylation and which, in the case of non-cyclic photophosphorylation, give the closest agreement with the stoichiometry of reaction III.

SUMMARY

Isolated chloroplasts from leaves of spinach, sugar beet, pokeweed, sunflower, and *Tetragonia expansa*, when prepared by techniques developed for spinach, had the ability to carry out three basic reactions found in spinach chloroplasts: cyclic photophosphorylation of either the flavin mononucleotide (FMN) or the vitamin K type, non-cyclic photophosphorylation, and assimilation of carbon dioxide to the level of carbohydrates.

Chloroplasts from all the species tested have also shown a capacity for photosynthetic phosphorylation coupled with the reduction of ferricyanide.

LITERATURE CITED

1. ALLEN, M. B., D. I. ARNON, J. B. CAPINDALE, F. R. WHATLEY, and L. J. DURHAM 1955. Photosynthesis by isolated chloroplasts. III. Evidence for complete photosynthesis. Jour. Amer. Chem. Soc. 77: 4149–4155.
2. ALLEN, M. B., F. R. WHATLEY, and D. I. ARNON 1958. Photosynthesis by isolated chloroplasts. VI. Rates of conversion of light into chemical energy in photosynthetic phosphorylation. Biochim. Biophys. Acta 27: 16–23.
3. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
4. ARNON, D. I., M. B. ALLEN, and F. R. WHATLEY 1954. Photosynthesis by isolated chloroplasts. Nature 174: 394–396.
5. ARNON, D. I. 1955. The chloroplast as a complete photosynthetic unit. Science 122: 9–16.
6. ARNON, D. I. 1958. Chloroplasts and photosynthesis. Brookhaven Symposia in Biology 11: 181–235.
7. ARNON, D. I. 1959. The conversion of light into chemical energy in photosynthesis. Nature 184: 10–21.
8. ARNON, D. I. and F. R. WHATLEY 1949. Factors influencing oxygen production by illuminated chloroplast fragments. Arch. Biochem. 23: 141–156.
9. ARNON, D. I., F. R. WHATLEY, and M. B. ALLEN 1954. Photosynthesis by isolated chloroplasts. II. Photosynthetic phosphorylation, the conversion of light into phosphate bond energy. Jour. Amer. Chem. Soc. 76: 6324–6329.
10. ARNON, D. I., M. B. ALLEN, and F. R. WHATLEY 1956. Photosynthesis by isolated chloroplasts. IV. General concept and comparison of three photochemical reactions. Biochim. Biophys. Acta 20: 449–461.

11. ARNON, D. I., F. R. WHATLEY, and M. B. ALLEN 1958. Assimilatory power in photosynthesis. Science 127: 1026–1034.
12. ARNON, D. I., F. R. WHATLEY, and M. B. ALLEN 1959. Photosynthesis by isolated chloroplasts. VIII. Photosynthetic phosphorylation and the generation of assimilatory power. Biochem. Biophys. Acta 32: 47–57.
13. AVRON, M., D. W. KROGMANN, and A. T. JAGENDORF 1958. The relation of photosynthetic phosphorylation to the Hill reaction. Biochem. Biophys. Acta 30: 144–153.
14. BENSON, A. A. and M. CALVIN 1950. Carbon dioxide fixation by green plants. Ann. Rev. Plant Physiol. 1: 25–42.
15. BROWN, A. H. and J. FRANCK 1948. On the participation of carbon dioxide in the photosynthetic activity of illuminated chloroplast suspensions. Arch. Biochem. 16: 55–60.
16. BURK, D. and O. WARBURG 1951. Ein-Quanten-Reaktion und Kreisprozesse der Energie bei der Photosynthese. Zeit. Naturforsch. 6b: 12–22.
17. GIBBS, M. and M. A. CYNKIN 1958. Conversion of carbon-14 dioxide to starch and glucose during photosynthesis. Nature 182: 1241–1242.
18. HILL, R. 1951. Reduction by chloroplasts. Symposia Soc. Exp. Biol. 5: 223–231.
19. JAGENDORF, A. T. 1958. The relationship between electron transport and phosphorylation in spinach chloroplasts. Brookhaven Symposia in Biology 11: 226–258.
20. KROGMANN, D. W., A. T. JAGENDORF, and M. AVRON 1959. Uncouplers of spinach chloroplast photosynthetic phosphorylation. Plant Physiol. 34: 272–277.
21. LUMRY, R., J. D. SPIKES, and H. EYRING 1954. Photosynthesis. Ann. Rev. Plant Physiol. 5: 271–340.
22. PFEFFER, W. 1900. Physiology of Plants. Clarendon Press, Oxford
23. SACHS, J. 1887. Lectures on the physiology of plants. Clarendon Press, Oxford
24. TREBST, A. V., H. Y. TSUJIMOTO, and D. I. ARNON 1958. Separation of light and dark phases in the photosynthesis of isolated chloroplasts. Nature 182: 351–355.
25. TOLBERT, N. E. 1958. Secretion of glycolic acid by chloroplasts. Brookhaven Symposia in Biology 11: 271–275.
26. VAN NIEL, C. B. 1949. The comparative biochemistry of photosynthesis. In: Photosynthesis in Plants, J. Franck and W. E. Loomis, eds. Iowa State College Press Pp. 437–495.
27. WARBURG, O. 1949. Heavy Metal Prosthetic Groups and Enzyme Action. Pp. 200–219. Clarendon Press
28. WESSELS, J. S. C. 1957. Studies on photosynthetic phosphorylation. I. Photosynthetic phosphorylation under anaerobic conditions. Biochim. Biophys. Acta 25: 97–100.
29. WHATLEY, F. R., M. B. ALLEN, L. L. ROSENBURG, J. B. CAPINDALE, and D. I. ARNON 1956. Photosynthesis by isolated chloroplasts. V. Phosphorylation and carbon dioxide fixation by broken chloroplasts. Biochim. Biophys. Acta 20: 462–468.
30. WHATLEY, F. R., M. B. ALLEN, and D. I. ARNON 1957. Cofactors of photosynthetic phosphorylation. Plant Physiol. 32 Suppl.: iii.
31. WHATLEY, F. R., M. B. ALLEN, A. V. TREBST, and D. I. ARNON 1958. Photosynthesis by isolated chloroplasts from different plants. Plant Physiol. 33 suppl.: xxvii.
32. WHATLEY, F. R., M. B. ALLEN, and D. I. ARNON 1959. Photosynthesis by isolated chloroplasts. VII. Vitamin K and riboflavin phosphate as cofactors of photosynthetic phosphorylation. Biochim. Biophys. Acta 32: 32–46.

ACTION AND TRANSMISSION SPECTRA OF PHYCOMYCES[1,2]

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In this paper measurements are presented of action spectra for the photoresponses of sporangiophores of Phycomyces and of the transmission spectra of the growing zone of single sporangiophores in stage IV b (7) and of the cell wall. Compared to other organisms, Phycomyces offers several definite advantages for such measurements. There are two distinct photoresponses, the growth response and the tropic response, and there are many indications, particularly the action spectra presented here, that these two responses are mediated by the same pigment. The growth response occurs under conditions of symmetric irradiation when the intensity varies with time, and the tropic response occurs with an irradiation program which may be constant in time, but asymmetric with respect to azimuth angle, i.e., the angle around the axis of the sporangiophore. Crudely speaking, the growth response measures the azimuthal summation of photoeffects, while the tropic response measures the azimuthal asymmetry. The combination of these two measurements permits an estimate of the influence of screening pigments lying *between* the receptors proximal to the incident light and those distal to it. Similarly, the measurement of transmission spectra of the intact sporangiophore and of the cell wall permits some conclusions with respect to the absorbing materials present in various locations.

Another advantage of Phycomyces is that the optics of the system are relatively simple. The sensitive region closely approximates a cylindrical lens with uniform refractive index. The importance of the dioptric properties of this lens for the tropic effect was clearly demonstrated 40 years ago by Buder (2), who showed that immersion of the specimens in a medium with refractive index higher than that of the cell contents reversed the sign of the tropic response. Thus, the dioptric properties, both refractive and reflective, and not scattering or absorption have the decisive influence in producing the azimuthal asymmetry in the sporangiophore.

Two years ago, Curry and Gruen (10) discovered that the tropic sensitivity of the sporangiophores extends deep into the ultraviolet region and that the tropic response reverses sign around 300 mμ. The reversal has a simple explanation. There are screening substances in the growing zone which, for wavelengths shorter than 300 mμ, effectively shield the distal side of the sporangiophore from the incident light. For these short wavelengths, then, the sporangiophore can be stimulated truly unilaterally, in contrast to the visible region, where both sides are stimulated, the distal more than the proximal, when the sporangiophore is irradiated unilaterally.

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METHODS

CONCEPT OF "VISUAL" PIGMENT Any response of an organism to light may be expected to involve at least one substance which undergoes a *photochemical reaction* which ultimately, through a more or less complicated chain of events, leads to the observable response. This substance will be referred to here as the "visual" pigment. The general purpose of making measurements of action spectra is to obtain information regarding the absorption spectrum of this visual pigment. However, the action spectrum *of the effect* may differ from the action spectrum *of the pigment* because of the intervention of screening pigments or of self screening. These difficulties will be discussed after presenting the data. The action spectrum of the pigment is usually equated with its absorption spectrum. This equation is not necessarily valid, since the quantum yield may vary with wavelength.

EQUAL RESPONSE VERSUS EQUAL FLUX ACTION SPECTRA Visual pigment absorbs different wavelengths with different absorption coefficients. This variability may be exhibited either as a difference in the *response* at various wavelengths under conditions of equal energy or quantum flux, or as a difference in *flux* needed to produce equal responses. Both methods have been used by various authors and both are likely to disclose at least the gross features of the absorption spectrum. The equal response method, however, seems preferable for quantitative work because it does not involve the uncertainty of translating differences in response into differences in amount of the primary photochemical product. The measurements presented here are based entirely upon the principle of equal response.

NULL VERSUS FINITE RESPONSE The next question concerns the choice of size of the "equal response". If there were no perturbations from side effects, such as screening, any size response should give the same action spectrum. The perturbations show up in a different dependence of the response on the flux at different wavelengths. A clear example of this may be found in the work of Shropshire and Withrow (16)

on the action spectrum of phototropic tip-curvature of Avena. Such perturbations can be interpreted properly only if a great deal of information is available besides the action spectrum. Quite aside from these perturbations, the question of the amount of response which should be chosen to obtain the most accurate measurements still remains. One condition is that the response should vary as strongly as possible with the flux. Another condition, well known in physical measurements, is that the response should vary symmetrically with the flux. This condition is best realized if the response is a null response, varying symmetrically between negative and positive values for small deviations from the null. Such a method eliminates perturbations arising from variability in sensitivity between specimens.

GROWTH RESPONSE NULL DETERMINATIONS For growth response the condition of null response was realized by irradiating the specimen during alternate equal intervals of 5 minutes from a standard light source and a monochromatic source. If these two sources appear equally bright to the specimen, there will be no growth response. This is the null condition. If the standard light appears brighter than the test light, there will be a periodic growth response whose maximum occurs about 7.5 minutes after the standard light is turned on; i.e., in the middle of the test light period. If the test light appears brighter, there also will be a periodic growth response, but with a phase shift of 180°.

The symmetry of the irradiation was assured by rotating the specimen continuously at 2 rpm. The arrangement for doing this while maintaining accurate positioning has been described previously (9). The speed of 2 rpm is sufficiently fast to eliminate tropic effects and sufficiently slow to avoid mechanical problems. Figure 1 illustrates the irradiation program and the actual responses from minute to minute under various conditions of relative size of the test and standard stimulus. The method by which the flux producing the null response was interpolated from a series of such measurements is illustrated in figure 2 and explained in the figure legend.

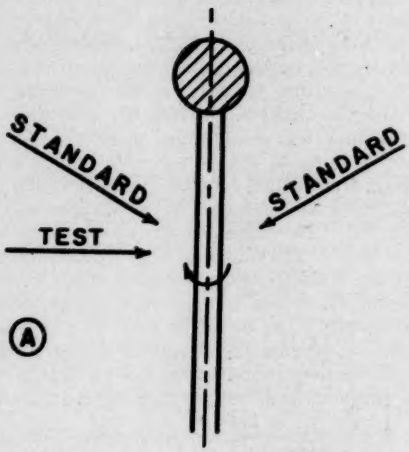
TROPIC NULL DETERMINATIONS For the tropic response the null method is more obvious and has been used by previous authors[4] (5). The standard light impinges from one side and is matched against the monochromatic test light. The monochromatic light impinges from the other side, if test and standard produce tropic effects of the same sign, and from the same side, if they produce tropic effects of opposite sign. It is important that the two beams of light do not come in at an angle of 180° from each other, but at a lesser angle. If the angle is 180° and the sources are of equal effectiveness, the specimens are indifferent phototropically; i.e., they are at equilibrium in whichever direction they grow (11). Another condition, only imperfectly realized in these experiments, is that the irradiation ought to be symmetric with respect to the vertical in order to eliminate perturbations from geotropism (12). Arrangement of the light sources is shown diagrammatically in figure 3. The equilibrium intensity of the test beam was determined by changing the intensity until the equilibrium position was obtained and maintained for 3 hours under continuous irradiation. Variations in the direction of growth of a single specimen during constant irradiation and variations between specimens amount to ± 10°. This variation sets a limit to the accuracy in a single determination of the matching quantum flux of ± 10 %. The matching quantum flux was determined for one to three sporangiophores at each wavelength. The sensitive zone of each sporangiophore was continuously centered to within ± 2 mm in all directions from the center of the field to eliminate the effect of small intensity gradients in the field.

EQUIPMENT FOR STIMULATION The standard stimulus was provided by a tungsten lamp with a Corning heat filter [1-69] and a Corning blue filter [5-61] arranged in tandem. Arrangement of the light source, stage, microscope, etc., as well as the intensity scale, have been described in detail previously (11). For most of the measurements the intensity of this irradiation was $I = -8$. Unilateral $I = -8$ corresponds in effectiveness to a monochromatic flux at 440 mμ of 0.022 μw/cm².

The test stimulus was provided by a uniform field (1.5 × 2.0 cm) of monochromatic flux obtained 30 cm from the exit slit of a Beckman DU spectrophotometer. Only the light sources and the dispersing elements of this instrument were used. The tungsten lamp was used for wavelengths longer than 330 mμ and the hydrogen lamp for shorter wavelengths. The intensity of the monochromatic flux is proportional to the square of the slit width (since entrance and exit slit vary in parallel) and this intensity control, after verifying its validity, was used in preference to neutral filters. The *maximum* half-intensity bandwidth, as calculated from Beckman instrument data, was 5 mμ. In most cases it was considerably smaller. The wavelength calibration was checked by isolating the bright lines from a medium pressure mercury arc source. The test light impinged horizontally from one side only, while the standard light impinged 60° from the vertical, from one or both sides.

CALIBRATION OF ENERGY FLUX The plate current, suitably amplified, of a calibrated RCA 935 phototube was used to measure the monochromatic flux. The sensitivity of the phototube was determined from 240 to 405 mμ by the Experimental Tube Division of RCA, using the strong mercury lines. For longer wavelengths the tube was calibrated by comparison with a Kipp thermopile and Leeds and Northrup galvanometer system, which in turn had been calibrated against a secondary irradiation standard. From the sensitivity data the quantum fluxes for a 0.10 mm slit were tabulated at 5 mμ intervals.

[4] See addendum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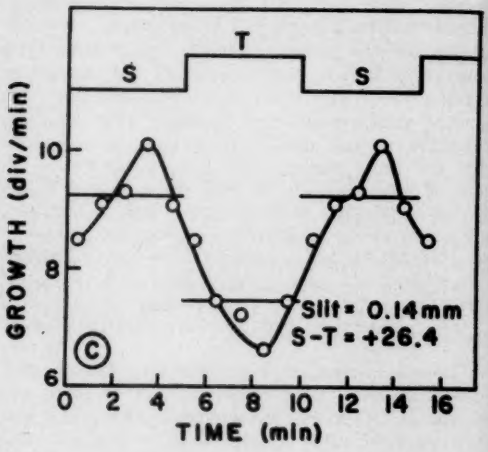
TEST
STANDARD
A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T
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6
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S-T = +26.4
C
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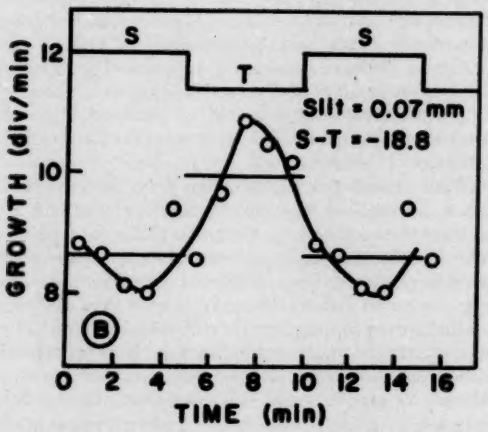
TIME (min)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T
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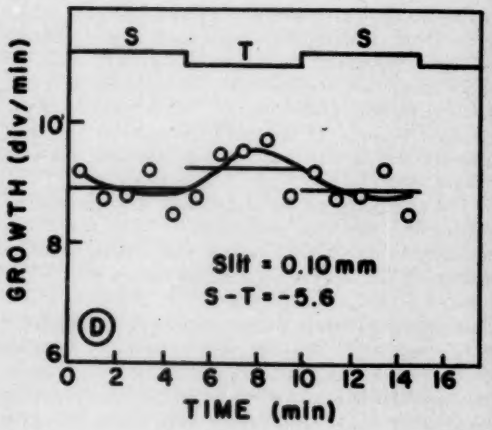
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T
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8
6
Slit = 0.10mm
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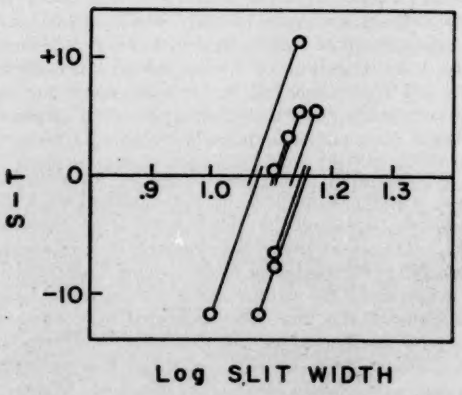
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-10
S-T
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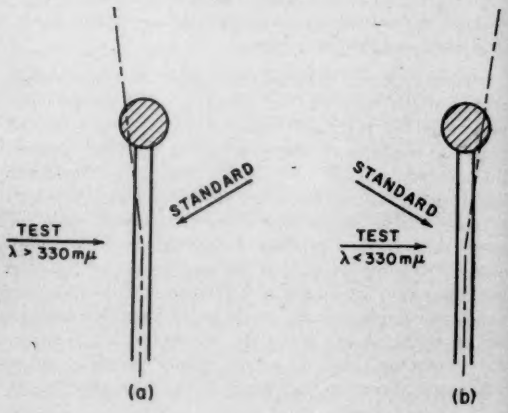
1.0
1.2
1.3
Log SLIT WIDTH


TEST
λ > 330mμ
STANDARD
(a)
STANDARD
TEST
λ < 330mμ
(b)

TRANSMISSION SPECTRA *Mounting specimens* After determining the action spectra, which showed pronounced peaks, an attempt was made to detect the visual pigment directly by the absorption of light during its passage across the sporangiophore. The dioptric properties of the specimens in air make it difficult to arrange a suitable geometry of the light paths in the optical system. These difficulties are much reduced by immersing the specimens in a medium of matching refractive index which is also transparent and non-toxic for the specimen. Pure fluorochemical FC-43, perfluorotributylamine, (Minnesota Mining and Manufacturing Co.) closely approximates these conditions. Specimens completely submerged in it grow at a normal rate for many hours and give normal growth responses. Its refractive index is 1.29 which is close to that of the cell contents (1.34 from our measurements).

It is convenient to work with sporangiophores which have been severed from their mycelium in order to mount them on a photographic plate or a microscope stage. Such specimens retain their turgor for many hours but do not grow when mounted in air or FC-43. Gruen (15) discovered that isolated sporangiophores grow and respond normally for many hours when their base is immersed in water. We have found that when the growing zone is in contact with water, growth ceases quickly and turgor is lost. Thus, for the present purposes, it was desirable to have specimens with their feet in water and their tops in FC-43. Microchambers (figure 6a) were constructed with two partitions, separated by silicone grease. An isolated sporangiophore is placed with the top half in one partition, where it is immersed in FC-43, and the lower half in the other partition, where it is immersed in water. Such specimens grow at a normal rate of 3 mm/hr with little or no lag due to shock of immersion, give normal growth responses, and can be observed continuously under a high power microscope. This arrangement permits a check whether or not the transmission of non-growing isolated specimens differs from that of growing specimens. No differences were found. Thus, the non-growing specimens give a valid comparison picture to the action spectra, which naturally require growing specimens.

Numerous methods for the transmission measurements were tried and the two relatively most successful ones are presented. For both methods the specimens were immersed in FC-43.

SHADOWGRAPH METHOD The specimen is placed directly on the emulsion of a photographic plate and irradiated from above with nearly parallel monochromatic light. The shadowgraph thus produced is evaluated densitometrically. Since the specimens are only 0.1 mm in diameter and the plates have to be scanned across the specimen, this method requires fine grain emulsion and high resolution densitometry. The method is laborious in requiring a new specimen for each wavelength and densitometric calibrations for each wavelength setting. It is particularly useful in the ultraviolet since no optics are involved in taking the shadow graphs; the method is informative with regard to the amount of light scattered by the cell contents under various angles.

Turgid, isolated sporangiophores were placed on Kodak medium lantern slide plates, imbedded in FC-43, covered with quartz coverslips supported by 0.5 mm spacers, and exposed to monochromatic flux. FC-43 can be left in contact with a photographic emulsion for long periods without affecting it. Exposures were such that the half-intensity bandwidths were 5 mμ or less and the background blackening was at the top of the linear range for a plot of optical den-

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FIG. 1 (*upper 2 rows*). Growth response, null method. A. Directions of irradiation. The specimen is rotated at 2 rpm to make the irradiation azimuthally symmetric. B, C, and D. Growth speeds during successive 1 min intervals. Above each response curve the irradiation program is indicated. The points in the response curves represent averages over three successive cycles. Average values during the first half of the cycle are repeated to facilitate the drawing of a smooth curve through the points. In B, with the slit of the monochromator at 0.07 mm, the test light is weaker than the standard light. The response curve has a minimum near the middle of the standard irradiation. In C, with the slit of the monochromator at 0.14 mm, the test light is stronger than the standard light. The maximum of the response occurs near the middle of the standard irradiation. In D, with a slit of 0.10 mm, the two irradiations are nearly matched. To evaluate the response, the total elongations during each of three standard (S) and three test (T) periods were determined. The difference between these two quantities, S-T, is negative in B, positive in C, and ideally zero at the growth response null. This condition is approximated in D. The horizontal lines are the average growth speeds during standard and test periods.

FIG. 2 (*lower left*). Interpolation method for determining the growth response null. The quantity S-T, obtained as explained in figure 1, is plotted versus the logarithm of the slit width. From the total of all measurements at all wavelengths it was found that S-T near the null varies linearly with the logarithm of the slit width, with a slope 3. Assuming this relation, every experimental point in this graph may be used to estimate a slit width giving null response. These estimates were averaged for five to ten sporangiophores at each wavelength to estimate the matching quantum flux.

FIG. 3 (*lower right*). Arrangement of the light sources for determining the tropic null. The dotted line indicates the direction of equilibrium. Due to geotropism, this direction is slightly closer to the vertical than the bisector of the two directions of growth produced by each of the sources separately.

FIG. 4. Shadowgraph method for determining transmission spectra. The specimen, immersed in FC-43, was placed directly on the emulsion of the photographic plate and irradiated with a parallel beam of monochromatic light. The shadowgraph was obtained at 380 mμ and is enlarged 45 x. Light bands at the margins of the sporangiophore are due to the residual difference between the refractive indices of the medium (1.29) and the cell contents (1.34). The residual lens effect produces an increased intensity in the center of the shadowgraph calculated to be 14 %.

sity (O. D.) versus the logarithm of exposure. Exposures were varied by changing exposure times at constant intensity. The gamma (γ) of the lantern slides and suitable exposure times were determined for the range from 240 to 540 mμ, in 10 mμ steps over most of the range and 5 mμ steps near the maxima, minima, and inflection points of the action spectra. The plates were scanned 1 to 2 mm below the sporangium with a microdensitometer capable of measuring fields 1.2 × 25μ (figs 4 and 5). The same instrument was used to determine the γ of the plates.

Below 300 mμ so little light is transmitted (due to absorption) that background and specimen cannot be compared in a single photograph. For these points longer exposures were made until the transmitted light gave appreciable blackening and the O.D. was estimated by comparing the exposure times used with those needed to produce similar background blackening. At very high O.D.'s this procedure is subject to errors from scattering, so that only lower limits of the O.D. can be estimated.

DIRECT METHOD In the second method the total transmitted light is measured *directly*. A 2 mm section of the exit slit of the monochromator is imaged with a 5 in focal length lens on the growing specimen and is carefully aligned parallel to the specimen axis. A slit width is chosen such that the image of the slit is narrow compared to the sporangiophore diameter. The light paths are therefore collimated to nearly the same degree as in the shadowgraph method. The total transmitted light (the undeviated and a large proportion of the scattered light) is collected by a microscope objective and measured by a photometer whose sensitive element intercepts the image of the observed section of the specimen. This method is convenient since the entire wavelength range can be scanned within a few minutes. It is also more accurate than the shadowgraph method since it does not require elaborate calibrations at each wavelength and avoids interspecimen variability. This gain is offset, however, by extreme requirements in alignment, focusing, positioning and by chromatic aberrations in the optics. These factors introduce errors which seem to be particularly bad in the near-ultraviolet. The method could not be used at all below 400 mμ.

The specimen, contained in a microchamber on the mechanical stage of a Reichert microscope, was aligned using the regular microscope optics (fig 6b). The eyepiece and top part of the tube were then removed and the image produced by a 45x objective projected via a totally reflecting prism onto the sensitive surface of a 1P21 photomultiplier tube of an Eldorado photometer. The specimen was moved in and out of the beam by fine motions of the stage controlled by reproducible front and back stops. At each wavelength setting the background was adjusted to zero by varying the fine sensitivity control of the photometer and the O.D. of the specimen in the growing zone alone or below the growing zone was determined directly by moving it into the beam. Under these conditions any light transmitted directly or scattered within the specimen into a forward angle less than about 45° was intercepted by the objective lens (N.A. = 0.65) and projected onto the sensitive surface.

RESULTS

GROWTH RESPONSES AND ADAPTATION AT DIFFERENT WAVELENGTHS The question of whether or not the specimens exhibited any qualitative differences in their responses to different wavelengths, aside from the reversal of phototropism, was examined first. Figure 7 shows that the growth responses elicited by stimuli of different wavelengths are exactly comparable, even deep in the ultraviolet where the stimulating light penetrates only a short distance into the specimen. Furthermore the ultraviolet is comparable to the visible in its adapting effects. As is well known (11), growth responses depend upon the ratio of the stimulus size to the level of adaptation, and the latter, at equilibrium, is proportional to the intensity of the adapting light. Light of any wavelength

was found to be interchangeable for either stimulus or adaptation, providing the intensity was adjusted by the calibration factor corresponding to the growth response action spectrum.

TROPIC RESPONSES AT DIFFERENT WAVELENGTHS In contrast to the growth response there are gross qualitative changes in the tropic response at different wavelengths. The most obvious of these is the reversal of the sign of the tropic response below 300 mμ.

In the visible a short unilateral stimulus of *moderate* size produces a tropic response for which the time of onset is very nearly the same as that of the growth response (3). A *strong* unilateral stimulus produces at best a feeble and somewhat delayed tropic response. This phenomenon was discovered by Castle (4) and named *phototropic indifference*. Castle interpreted it by assuming that a strong stimulus gives a saturated growth response on both the proximal and the distal side and, therefore, no differential between the two sides.

Below 300 mμ, the distal side is shielded. Under these conditions *any* stimulus capable of producing a growth response should also give a tropic response. This is indeed the case. Below 300 mμ a strong, short unilateral stimulus gives a strong, transient, negative tropic response. The response begins around 4 minutes after the stimulus and results in a maximum tilt of 70° or more. The maximum *rate of tilting* is about 8°/min and occurs at 5 to 7 minutes after the stimulus. The *maximum tilt* occurs at 11 to 15 minutes and the tilt is back to zero at about 30 minutes. These are precisely the characteristics expected for a strictly unilateral growth response.

Similarly, continued unilateral stimulation at 280 mμ gives a continued negative tropic response, with a maximum rate of tilting greater than 20°/min.

For specimens immersed in FC-43 the match of refractive indices is so close that no phototropic response occurs even after prolonged unilateral irradiation with blue light. Irradiation with wavelengths shorter than 300 mμ, however, produces a strong negative tropic reaction. This is to be expected, since the specimen is stimulated exclusively on the side proximal to the impinging light.

GROWTH RESPONSE AND TROPIC RESPONSE ACTION SPECTRA Figure 8 shows the results of these measurements, obtained by the null method described above. The matching intensity was the same (or a small multiple) in all cases, $I = -8$; i.e., close to the lower end of the "normal range" (11). For each wavelength measured, the ordinate gives the reciprocal quantum flux which matches the standard light. For wavelengths below 300 mμ the matching reciprocal quantum fluxes for the tropic responses are entered as negative values to indicate that the test light impinged from the same side as the standard light.

TRANSMISSION SPECTROSCOPY OF GROWING ZONE As pointed out above, the shadowgraph method was the only one available below 400 mμ. This method was also used for longer wavelengths but was less precise than the direct method. The precision for the shadowgraph method was ± 0.1 O.D. unit, while for the direct method the values were reproducible for a given specimen to within 0.01 O.D. unit. In figure 9 the measurements in the ultraviolet are given both for intact sporangiophores and for cell walls from which the contents had been squeezed out.

The O.D. rises sharply slightly above 300 mμ (13). Dennison has identified the principal substance in the sap which absorbs in the ultraviolet as gallic acid. Since the onset of absorption of this substance agrees with our O.D. findings, the absorption curve of gallic acid, adjusted to equal O.D. at 290 mμ is given in figure 9. The match between the two curves is remarkably good above 250 mμ. It is poor below 250 mμ, where the expected O.D. of gallic acid decreases while the observed O.D. continues to remain high. Presumably, other substances contribute to the O.D. in this region.

The concentration of gallic acid needed to produce the indicated O.D. in a pathlength equal to the diameter of the sporangiophore (100 μ) is about 5 mg/ml. This is close to the value estimated by Dennison for the concentration of gallic acid in the extracted *sap* of *whole* sporangiophores (13). These facts strongly suggest that the internal screen, at least near the neutral point, is gallic acid.

For later discussion of the inversion of phototropism the O.D. contributed by gallic acid in the critical region between 300 and 330 mμ is given in table I.

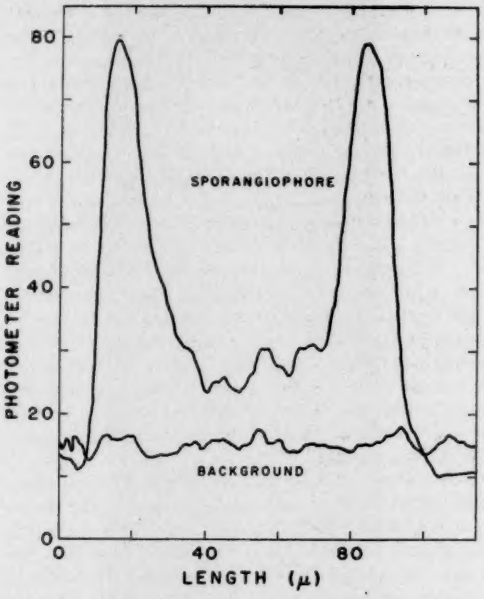
The absorption of the cell wall (fig 9) shows the absorption peak reduced by a factor of about ten. This is believed to be due to residual contents not eliminated during the squeezing process.

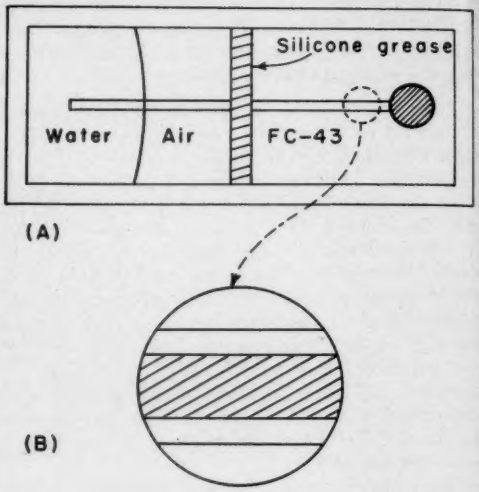
In the visible the results were quite variable, and are therefore not plotted. A few fairly regular features could be discerned, however. Generally, a background O.D. of about 0.1 was found. This is presumably due in large part to nonspecific absorption and in small part to reflection losses and residual scattering not collected by the microscope objective. Superimposed on this background is a slight absorption, amounting to a maximum O.D. of about 0.03, beginning at about 530 mμ, and giving maxima around 500, 460 and 430 mμ. In sporangiophores grown on a lactate medium, this characteristic absorption is much stronger, contributing in some cases O.D.'s between 0.1 and 0.2. It is likely that the substance con-

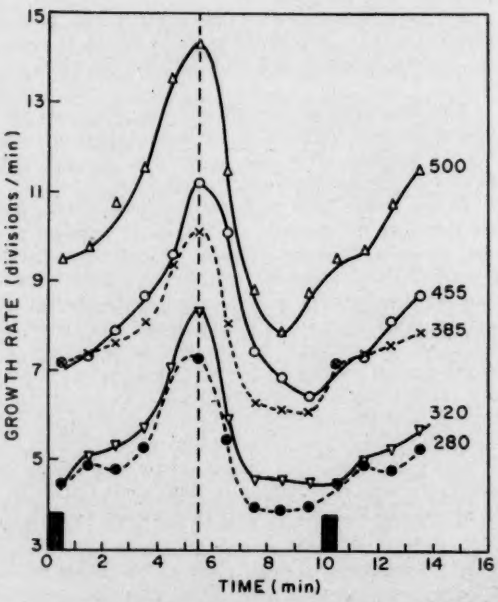
TABLE I

OPTICAL DENSITY OF GALLIC ACID (5MG/ML FOR 100 μ PATHLENGTH)

λ	O.D.
300	0.70
310	0.24
320	0.05
330	0.006







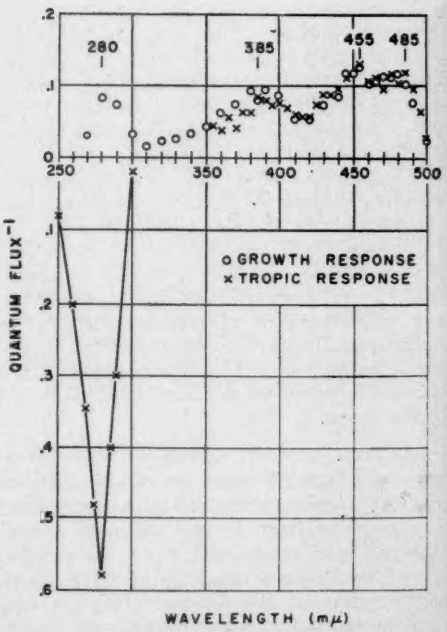


FIG. 5 (*upper left*). Microdensitometer tracing (1 mm below the base of the sporangium) across the sporangiophore of the original shadowgraph of figure 4. Irregularities in the tracing are due to grain in the emulsion. The principal point exhibited is the sharpness of the edge of the sporangiophore, indicating that scattering does not affect the optics of the sporangiophore appreciably.

FIG. 6 (*upper right*). Microchamber for direct transmission measurements. A. Top view of chamber. B. The field imaged by the microscope objective onto the sensitive element of the photometer, showing the image of the slit of the monochromator (hatched area) in relation to the specimen.

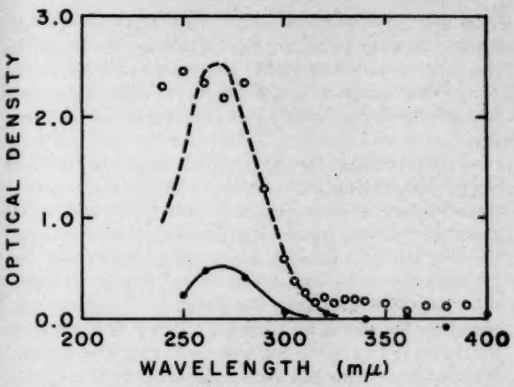


FIG. 9. Transmission spectra of the growing zone of the sporangiophore and of the primary cell wall in this region. The dotted line is the optical density of gallic acid (5 mg/ml 100μ pathlength). Open circles denote the O.D. of intact growing zone and solid circles the transmission curve of the cell wall with imperfectly extruded cell contents. The data are corrected for the lens effect mentioned in the legend to figure 4.

tributing these three peaks is β-carotene, since β-carotene can be isolated from the extracts and has similar absorption peaks, except for a slight wavelength shift.

The cell wall had no characteristic absorption (less than 1 %) in the visible and near-ultraviolet, even when the specimens had been carefully dark adapted before extruding the cell contents.

No bleaching by blue light could be observed, even when the specimen was growing and responding normally and had been thoroughly dark adapted.

DISCUSSION

The principal aim of this discussion is to determine what can and cannot be concluded with respect to the action spectrum of the visual pigment. The following are the main points in our data:

I. The growth and tropic action spectra are identical from the upper limit at 500 mμ to 360 mμ, and show peaks at 385, 455, and 485 mμ.

II. Below 300 mμ the tropic action spectrum is a negative image of the growth action spectrum, enlarged at the peak (280 mμ) by a factor 6.5.

III. The cell wall does not show a characteristic absorption.

IV. Beyond 400 mμ the O.D. of the growing zone of a sporangiophore, whether non-growing, or growing and reactive, whether dark adapted or not, shows no obvious peaks which could be correlated with those of the action spectra.

V. The O.D. begins to rise sharply near the inversion point and reaches a maximum (about 2.5) at the 280 mμ peak.

These findings are discussed in terms of the following postulates which are somewhat weaker than those originally proposed by Blaauw (1):

Growth responses are transient and occur as a result of *changes* in the intensity of irradiation. A positive intensity transient (a step-up or a pulse-up) produces a positive growth response. Any phototropic effect in Phycomyces is conditioned by a *bilateral asymmetry* of light energy *absorbed*. (A puzzling feature of the tropic response to continuous unilateral irradiation is that it is not transient, like the growth response to a step-up in the intensity, but persists beyond the time at which the growth response transients disappear. This has been discussed in detail by Cohen and Delbrück (8)).

For evaluating the effectiveness of light absorbed we have to take into account its precise azimuthal distribution (6). Thus, for unilateral irradiation in air where the total light absorbed on the proximal side probably slightly exceeds the amount absorbed on the distal side, the distal absorption is nevertheless more effective due to the advantage conferred upon the distal side by the focusing effect. We have no a priori measure of the magnitude of this advantage, but will attempt to evaluate it from our data. Let us say that the focusing effect in air gives the distal side the stimulus 1+a when the proximal side receives unit

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FIG. 7 (*lower left*). Growth responses at different wavelengths. The specimens were rotated at 2 rpm and continuously adapted by irradiation with standard light. They were stimulated every 10 min for 30 sec with monochromatic light of suitable intensity. The points are averages for three cycles. The growth responses obtained at different wavelengths are similar. Maximum response occurs at 5.5 min after the stimulus. The response time is independent of the average growth rate and of the size of the response. The correlation between wavelength and average growth rate of the specimen is accidental.

FIG. 8 (*lower right*). Action spectra for growth response and tropic response. For the tropic response data, the matching quantum fluxes were determined with a precision of ± 10 %. The precision of the growth response data was somewhat better. The maxima at 280 and 385 mμ and a broad region of high sensitivity between 440 and 490 mμ are unambiguous. The dip between the two maxima at 445 and 485 mμ could be due to a combination of experimental errors and screening effects, as explained in the text. Otherwise the action spectra, where they coincide, are believed to represent faithfully the action spectrum of the visual pigment. The distortions below 300 mμ are due to internal screening by gallic acid. The growth response peak at 280 mμ is low by *at least* a factor two, as compared to the peak in the action spectrum of the visual pigment (see text).

stimulation. Superimposed on this advantage is a factor, 1−b, accounting for any attenuation of the light in its passage across that part of the sporangiophore between the proximal and the distal receptors. Such an attenuation could be due to the visual pigment itself and to other substances. The focusing advantage a is presumably independent of wavelength (neglecting dispersion), while the attenuation b depends strongly on the wavelength. In the visible the attenuation is small compared to unity, according to point 4 above. Since the reversal occurs between 320 and 310 mμ, where the total O.D. is still small compared to unity and the absorption due to gallic acid contributes an O.D. between 0.05 and 0.24 (see table I), we conclude that the focusing advantage is also small compared to unity. In the visible, then, both a and b are small and we can neglect squared terms. Thus, the ratio of the effects on the two sides is

$$\text{distal : proximal} = (1+a-b) : 1.$$

The effects of various types of screening on the two action spectra may now be considered systematically.

I. Internal Screen, Attenuation Slight, $b<<1$ The growth action spectrum will not be affected, since it compares the sum of the effects on proximal and distal sides, standard versus test. Screening and focusing contribute only second order correction terms.

In contrast, the tropic action spectrum may be affected, since the null condition requires that the differentials are matched. Let the quantum flux of the standard light and also the absorption coefficient of the visual pigment for this light be unity. Let the quantum flux of the test light at equilibrium be x and the absorption coefficient of the visual pigment for this light be β. Let b_s and b_λ be the attenuations of standard and test light. Then the effects on the two sides will be:

standard side	$1 + \beta\, x(1+a-b_\lambda)$
test side	$\beta\, x + 1+a-b_s$

For tropic equilibrium, these two expressions must be equal. This gives for the action spectrum $1/x$ the expression

$$1/x = \beta\,(a-b_\lambda)/(a-b_s).$$

This equation says that the action spectrum and the absorption spectrum of the screening pigment will be in an inverse relation to each other. The action spectrum $(1/x)$ will have a dip where the screening pigment (b_λ) has a maximum. The reason for this is obvious: the screen increases the *intensity* differential where its spectrum has a maximum; it thereby *reduces* the advantage conferred upon the distal side by the focusing effect.

This effect is appreciable only where b_λ is comparable to a. In our data the effect is obvious as the inversion point is approached. As the O.D. of the screen rises and becomes comparable to the focusing advantage the tropic action spectrum drops off to zero.

Equally significant is the fact that we find no such screening effect in the visible as evidenced by the strict parallelism of the two action spectra (point 1). This indicates that there is no internal screening affecting appreciably the advantage due to focusing.

Self-screening by the visual pigment, if it is slight, has qualitatively similar effects. It, too, will not affect the growth action spectrum, but will affect the tropic action spectrum inversely, when it is comparable to the focusing advantage. Again we can conclude from the parallelism of the two action spectra that self-screening in the visible is negligible compared to the focusing advantage (less than 10 % of the latter). In sum, we conclude that the internal attenuation across the sporangiophore in the visible, both due to self- and foreign-screening, is negligible.

II. Internal Screen, Attenuation Strong, $1-b<<1$ The growth action spectrum will be affected by a factor two, since the standard light acts on both sides while the test light reaches the proximal side only. To match the standard light, therefore, the test light has to provide a quantum flux twice higher than corresponds to the absorption coefficient of the visual pigment, and the action spectrum is low by a factor two compared to the visual pigment absorption spectrum.

For the tropic action spectrum the situation is quite different. Consider a tropic equilibrium when visible light impinges from the same side as light of, say, 280 mμ. The visible light acts at the rate one on the proximal side and 1+a on the distal side. The ultraviolet acts on the proximal side only. To balance the asymmetry in the stimulation produced by the standard light it must act at the rate a. Thus, in such a combination, the ultraviolet does not actually match the standard light in its direct effect on the visual pigment, but only compensates the differential of this effect across the sporangiophore. Therefore, the quantum flux needed for tropic balance is low by the factor a, and the action spectrum high by the factor 1/a.

Combining these two corrections we are now in a position to estimate a numerical value for the focusing advantage a. To do this we must raise the growth action value by the factor two and compare it then to the tropic action value, which is 1/a times larger. This gives for a the value 0.3.

III. External Screens Any screening substance located entirely external to the photoreceptors will affect the growth and the tropic action spectrum equally and increase the quantum fluxes needed to match the test stimulus. Thus the action spectra should show dips where the screens show peaks. Unfortunately, we do not know where the visual pigment is located and, therefore, also do not know which regions are to be considered external. The cell wall is certainly external and it does not show any characteristic absorption (point 3). However, this does not suffice as a justification for ignoring the possible

effects of external screens. It may well be that β-carotene and gallic acid lie, at least in part, external to the visual pigment. The distortions from this source could be appreciable. In the ultraviolet, if only 10 % of the gallic acid were located external to the photoreceptor it would cut off half the flux at 280 mμ and make both action spectra low by a factor two. In the blue, if all the blue absorbing pigments as measured by the total O.D. were entirely external to the photoreceptor, half on each side, the action spectra would be depressed by an O.D. of 0.015. This depression would affect the action spectra by 3 to 4 % at its absorption peaks and might be responsible for some of the dip in the action spectra separating the peaks at 455 and 485 mμ.

The principal conclusions regarding the action spectrum of the visual pigment of Phycomyces are the following:

In the visible and near-ultraviolet both action spectra give a rather faithful image of the visual pigment action spectrum, not distorted by internal. external, or self-screening. Distortions from internal screens are entirely negligible. External ones might give distortions of a few percent; hardly noticeable within the accuracy of our measurements. The only significant region that might be affected by this correction is the dip separating the maxima at 455 and 485 mμ.

The total absorption of light in its passage between the photoreceptors on the two sides of the sporangiophore must be less than 10 % of the focusing advantage. Since this is less than the directly measured attenuation across the whole sporangiophore and since the cell wall has no measurable O.D., we must infer that the measured attenuation is due to pigments located peripherally but inside the cell wall. This makes it seem likely that the photoreceptors are not located at the cell wall but some distance inside. Perhaps they lie at the interface between protoplasm and vacuole or at some interface inside the protoplasm.

Regarding the chemical nature of the visual pigment we can rule out β-carotene with some confidence, because of the peaks at 280 and 385 mμ in the action spectra. Various flavin compounds and other substances mentioned in the literature (14) whose absorption spectra show greater similarity with the action spectra are possible candidates. With the action spectrum given it would seem hopeful to isolate the visual pigment and identify it by chemical means. Our attempts in this direction have not yet been successful. The O.D. produced by visual pigment must be very small; it does not show up in the transmission measurements, and self-screening does not affect the differential across the sporangiophore responsible for the tropic reaction.

The discussion of the interrelationship between the action spectrum of the growth response and that of the tropic response illustrates how strongly both spectra, and particularly the tropic spectrum, can be modified by screening pigments. In Phycomyces there happens to be no appreciable distortion in the visible, though a very strong one below 350 mμ. In Avena the work of Shropshire and Withrow (16) has shown that the tropic action spectrum is strongly affected by screening pigments even in the visible. To correct for these screening effects, it would be necessary to have a clear notion of the cause and magnitude of the bilateral asymmetry in the absence of screening pigments, similar to the notion of the focusing advantage in the case of Phycomyces. We do not have such a theoretical basis for Avena. We suspect that, here, too, dioptric effects are involved, since Ziegler (17) has shown that the phototropism for Avena reverses sign when the specimens are immersed in paraffin oil. We have confirmed this effect and have found that the extreme tip of the coleoptile, where most of the sensitivity resides, is sufficiently transparent to permit dioptric effects to be operative. A detailed growth action spectrum would help clarify this question.

Summary

Detailed action spectra of the growth response and of the tropic response of the sporangiophores of Phycomyces in stage IVb are given for 10 mμ intervals from 250 to 300 mμ and for 5 mμ intervals from 300 to 500 mμ. Both spectra have been determined by null response methods. The two spectra are identical in the visible and near-ultraviolet and show maxima at 280, 385, 455, and 485 mμ. Below 300 mμ the tropic response reverses sign and shows a peak at 280 mμ. This peak is 6.5 times larger than a corresponding peak in the growth action spectrum.

Transmission spectroscopic data in the growing zone have been obtained for single sporangiophores. In the ultraviolet these were obtained from shadowgraphs and in the visible by direct photometric measurements. In the ultraviolet a strong absorption is found, beginning near the phototropic inversion point and paralleling the absorption of gallic acid, a substance which had previously been detected by Dennison in the vacuole. In the visible region, transmission, including most of the scattered light, is never more than about 75 %. Only a minute part of these transmission losses can be due to absorption by the visual pigment. Most of the losses must occur peripheral to the visual pigment, but not in the cell wall.

The growth responses are qualitatively identical throughout the spectrum. The tropic responses, besides reversing sign below 300 mμ, are also stronger in this region than in the visible. These facts, as well as the quantitative relationship between the two action spectra, can be explained satisfactorily as mediated by the screening effect of an internal screen which is principally due to gallic acid.

Acknowledgements

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Addendum

A paper by G. M. Curry and H. E. Gruen has just appeared (Proc. Natl. Acad. Sci. 45: 797. 1959) in which action spectrum data for the phototropic response of Phycomyces are presented. The gross features of the results of these authors are similar to ours. Differences in detail may be accounted for by the method used by Curry and Gruen, involving mass cultures and the determination of balance points between the standard and the test light source which are set up 2 meters apart. This method, which we had tested and rejected, involves two disadvantageous features apparently not appreciated by the authors:

I. Mass cultures lead to mutual shading, and this produces spurious balance points near the middle of any series of cultures set up together.

II. The intensity ratio received from the two sources varies with position, as $(1+x)^2/(1-x)^2$, where x is the displacement from the center, taking the distance from the center to one of the sources as unit. This is a very rapid variation, approximately $1+4x$ for small displacements from the center. Thus, 2.5 cm displacement changes the ratio by 10%. A glance at table I of the cited paper shows that balance points in repeat measurements differed in some cases by as much as eight times this amount.

In view of these criticisms, the contention of Curry and Gruen of a double peak in the blue, characteristic of the absorption of β-carotene, cannot be considered as substantiated by their measurements.

Literature Cited

1. Blaauw, A. H. 1909. Die Perzeption des Lichtes. Rec. trav. bot. neerl. 5: 209–372.
2. Buder, J. 1918. Die Inversion des Phototropismus bei Phycomyces. Ber. deut. bot. Ges. 36: 104–105.
3. Castle, E. S 1930. Phototropism and the light-sensitive system of Phycomyces. Jour. Gen. Physiol. 13: 421–435.
4. Castle, E. S. 1931. Phototropic "Indifference" and the light-sensitive system of Phycomyces. Bot. Gaz. 91: 206–212.
5. Castle, E. S. 1931. The phototropic sensitivity of Phycomyces as related to wave-length. Jour. Gen. Physiol. 14: 701–711.
6. Castle, E. S. 1933. The physical basis of the positive phototropism of Phycomyces. Jour. Gen. Physiol. 17: 49–62.
7. Castle, E. S. 1942. Spiral growth and the reversal of spiraling in Phycomyces and their bearing on primary wall structure. Amer. Jour. Bot. 29: 664–672.
8. Cohen, R. and M. Delbrück 1959. Photoreactions in Phycomyces: Growth and tropic responses to the stimulation of narrow test areas. Jour. Gen. Physiol. 42: 677–695.
9. Cohen, R. and M. Delbrück 1958. Distribution of stretch and twist along the growing zone of the sporangiophores of Phycomyces during normal growth and during periodic illumination. Jour. Cellular Comp. Physiol. 52: 361–388.
10. Curry, G. M. and H. E. Gruen 1957. Negative phototropism of Phycomyces in the ultra-violet. Nature 179: 1028–1029.
11. Delbrück, M. and W. Reichardt 1956. System analysis for the light growth reactions of Phycomyces. Cellular Mechanisms in Differentiation and Growth. Rudnick, Ed. Princeton University Press. Pp. 3–44.
12. Dennison, D. S. 1958. Studies on phototropic equilibrium and phototropic-geotropic equilibrium in Phycomyces. Thesis, California Institute of Technology.
13. Dennison, D. S. 1959. Gallic acid in Phycomyces sporangiophores. Nature 184: 2036.
14. Galston, A. 1959. Phototropism of stems, roots and coleoptiles. Handbuch der Pflanzenphysiologie 17(1): 492–529.
15. Gruen, H. 1959. Growth and development of isolated Phycomyces sporangiophores. Plant Physiol. 34: 158–168.
16. Shropshire, W., Jr. and R. B. Withrow 1958. Action spectrum of phototropic tip-curvature of Avena. Plant Physiol. 33: 360–365.
17. Ziegler, H. 1950. Inversion phototropischer Reaktionen. Planta 38: 474–498.

OXIDATIVE PHOSPHORYLATION AND GLYCOLATE OXIDATION BY PARTICLES FROM SPINACH LEAVES[1]

ISRAEL ZELITCH AND G. A. BARBER

It has recently been reported (2) that oxidative phosphorylation occurs when organic acids of the citric acid cycle are supplied as substrates to particles isolated from the cotyledons of etiolated lupine seedlings. The ratios of the number of moles of orthophosphate consumed to the number of atoms of oxygen taken up (P/O ratios) were found to be as high as those previously reported only for animal tissues (7). Furthermore, Ohmura (13) has shown that particles obtained from green spinach leaves are also capable of carrying out oxidative phosphorylation although he did not determine P/O ratios. This observation suggested that spinach leaves might provide an active particulate system for further study. Accordingly, such particles have been isolated by a method modified from that of Ohmura and have been found to bring about phosphorylations with high P/O ratios when substrates such as pyruvate, citrate, L$_a$-isocitrate, *a*-ketoglutarate, succinate, fumarate, L-malate, and reduced diphosphopyridine nucleotide (DPNH) are used.

Glycolic acid oxidase, a riboflavin phosphate-linked enzyme which catalyzes the oxidation of glycolic acid to glyoxylic acid, was isolated some years ago in a highly purified form from extracts of spinach leaves (21) and has recently been crystallized (5). Experiments performed in this laboratory suggest that glycolic acid oxidase plays a role in the respiration of leaves in sunlight (19, 20). Some glycolic acid oxidase activity has now been found to be associated with the particles obtained from spinach leaves, and a search was accordingly made for evidence of oxidative phosphorylation with glycolate as the substrate. No significant activity was found. The effect of glycolate on the oxidation and phosphorylation of other substrates by these particles has also been investigated.

MATERIALS AND METHODS

PREPARATION OF PARTICLES: Spinach was purchased locally, and stored in a plastic bag at 5° C, occasionally for as long as several weeks, until used. Storage of leaves for this duration was found to have no effect on the activity of isolated particles. The younger pale-green leaves were taken unless otherwise noted. The midribs were removed, and a weighed quantity of leaf blade tissue was washed several times in water and chilled for a few minutes on ice before being ground in the cold room.

Grinding was done in a chilled mortar with washed sand, with the addition of two volumes of a grinding medium, similar to Ohmura's (13), of the following composition: 0.45 M sucrose, 0.05 M mannitol-borate buffer at pH 7.2, 0.03 M potassium citrate, 0.01 M sodium ethylenediamine tetraacetate (Versenate) at pH 7.5, and 0.05 M tris(hydroxymethyl)aminomethane chloride (Tris) at pH 8.3. The ground tissue, final pH 7.2, was squeezed through two layers of cheesecloth, and then centrifuged for 5 min at 600 × G at −5° C. The residue, consisting of chloroplasts and other heavy cell fragments, was discarded, and the supernatant fluid was centrifuged at 10,000 × G for 20 min. The residue of particles was gently suspended in a washing medium (1 ml per g of leaf) composed of 0.3 M sucrose, 2×10^{-4} M Versenate, and 0.05 M tris buffer at pH 7.5, and centrifuged at 10,000 × G for 20 min. The particles were finally taken up in the washing medium (1 ml per 1.8 g of leaf) for use in the experiments. Usually 0.9 ml of the suspension was used in each Warburg vessel. This is equivalent to 1.6 g of leaf tissue, and 0.5 to 0.8 mg of protein N per vessel. The suspension was still green in color, doubtless because of material originating in the chloroplasts.

MANOMETRY: Oxygen uptake was measured by conventional techniques in 15 ml Warburg vessels in an atmosphere of air. All of the substrates used were of the highest grade commercially available. Crystalline potassium pyruvate (9), L$_a$-isocitrate (17), and sodium glyoxylate monohydrate (15) were prepared in the laboratory. Cytochrome c from horse heart, yeast coenzyme concentrate, glucose-6-phosphate dehydrogenase, and Type III hexokinase from yeast were purchased from the Sigma Chemical Co. Substrates, cofactors, and the particles suspended in the washing medium were placed in the main compartment of chilled Warburg vessels. The center well contained 0.2 ml of 5 *N* KOH, and to the side arm glucose and an excess of hexokinase were added to convert the adenosine triphosphate (ATP) formed by oxidative phosphorylation into glucose-6-phosphate. After equilibration at 30° C for 5 min, at zero time, the contents of the side arm were tipped into the main compartment. The vessels were shaken at 120 oscillations per minute, and light was excluded with a black cloth. Under these experimental conditions, the rate of diffusion does not limit the oxygen uptake until a rate of 8 µl per minute is approached. At the end of the reaction period, the vessels were quickly transferred to an ice bath, and 1 ml of 3% perchloric acid was added to stop enzymic reaction.

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ANALYTICAL MEASUREMENTS: After centrifuging the reaction mixture, the orthophosphate remaining in the supernatant fluid was determined by a colorimetric phosphomolybdate method (12). In some experiments, the removal of inorganic phosphate by enzymic action was confirmed by assay of the glucose-6-phosphate formed (10).

Glyoxylate was determined by the method of Friedemann and Haugen (4) in experiments in which glycolate was the substrate. Total protein nitrogen was measured by Nesslerization after Kjeldahl digestion of the particles which had been washed with trichloroacetic acid.

RESULTS

OXIDATIVE PHOSPHORYLATION WITH ORGANIC ACIDS: Evidence is presented in table I that particles isolated from young spinach leaves contain a vigorous oxidative phosphorylation system with all the members of the Krebs citric acid cycle tested. In experiment 1, the value for *a*-ketoglutarate (theoretical P/O = 4.0) has probably been lowered by the accompanying oxidation of the succinate formed (theoretical P/O = 2.0). In contrast to the behavior of particles obtained by Pierpoint from tobacco leaves (14), these preparations are also able to oxidize fumarate. When fumarate, citrate, or pyruvate are the substrates, a lag in the rate of oxygen uptake was always observed for the first 10 to 15 min, presumably because the fumarase and aconitase activities are limiting, after which the rates of oxidation approached those of succinate and malate. Accordingly, the figures for the oxidation during 25 min given for fumarate, citrate, and pyruvate are minimal values.

TABLE I

OXIDATIVE PHOSPHORYLATION BY PARTICLES FROM SPINACH LEAVES WITH VARIOUS SUBSTRATES

EXPERIMENT NO.	SUBSTRATE	OXYGEN UPTAKE IN μATOMS	ORTHOPHOSPHATE ESTERIFIED IN μMOLES	P/O
1	Succinate	11.6	24.5	2.1
	Citrate	9.1	23.0	2.5
	L$_s$-Isocitrate	6.8	13.6	2.0
	a-Ketoglutarate	11.7	27.2	2.3
	Glycolate	5.2	0.4	0.1
2	Succinate	12.1	25.1	2.1
	Fumarate	5.7	16.2	2.8
	L-Malate	12.1	31.9	2.6
	Pyruvate*	5.9	15.7	2.7
	Glycolate	3.8	1.2	0.3
3	DPNH	2.1	5.3	2.5

* In the presence of a catalytic amount (2μmoles) of L-malate.

The reaction mixture in experiments 1 and 2 contained the following components in chilled Warburg vessels: sucrose, 300 μmoles; $MgSO_4$, 10 μmoles; substrate, 20 μmoles; potassium phosphate buffer at pH 7.0, 37 μmoles; yeast coenzyme concentrate, 1 mg; ATP, 2 μmoles; particles from 1.6 g of leaf tissue in 0.9 ml of washing medium; and water to make the final volume 2.0 ml. In the sidearm were placed glucose, 50 μmoles, and yeast hexokinase, 0.2 mg. The center well contained 0.2 ml of 5 *N* KOH. The reaction was stopped after 25 min at 30° C.

In experiment 3, all conditions were the same except that 6 μmoles of substrate and 19 μmoles of phosphate buffer were added, and yeast coenzyme concentrate was omitted. The reaction was terminated after 10 min.

All values are corrected for the slight uptake of oxygen (0 to 0.5 μatom) observed in a control vessel containing all components except substrate.

The P/O ratios obtained with succinate are equal to the highest values given in the literature (7) and serve as a criterion of the undamaged nature of the particles. This point is further demonstrated by the failure of added cytochrome c (0.5 mg per vessel) to stimulate the rate of oxidation of succinate. As has also been observed with particles from tobacco leaves (14), the rate of succinate oxidation is not influenced by added yeast coenzyme concentrate. As is demonstrated in table I, addition of fluoride is not necessary in order to demonstrate high P/O ratios (13); this indicates that the phosphatase activity of the spinach leaf particles is low.

Good agreement was achieved when the disappearance of orthophosphate was compared with the net increase in glucose-6-phosphate under conditions similar to those of table I. For example, in one experiment in which succinate was the substrate, 14.1 μmoles of orthophosphate were taken up (P/O = 1.7) and 13.0 μmoles of glucose-6-phosphate were produced (P/O = 1.6). No significant disappearance of orthophosphate has ever been observed in reactions carried out as described in table I but in the absence of substrate.

Although glycolate is oxidized by these particles, significant esterification of inorganic phosphate is not observed in the presence of this substrate. The enzyme which oxidizes glycolate could not be removed from the particles by further washing. Its activity in the particles represents about 15 % of that found initially in homogenates of the leaf; the first residue from centrifugation contained 25 % of the activity, and the remainder was present in the supernatant fluid.

REQUIREMENTS FOR PYRUVATE OXIDATION: The presence of a catalytic amount of malate, which presumably serves to produce an acetyl acceptor, is required to obtain good rates of oxidation of pyruvate (table II). This requirement is commonly observed in analogous systems prepared from other sources. The particles already possess a complement of coenzymes, but oxidation is further stimulated by adding DPN and coenzyme A (CoA). The high P/O ratio obtained, 2.7, approaches the highest figure, 3.0, observed with particles from other sources (7).

EFFECT OF AGE OF LEAVES ON OXIDATIVE PHOSPHORYLATION BY ISOLATED PARTICLES: Particles obtained from older spinach leaves, that is, leaves inserted lower on the stem than those hitherto discussed,

TABLE II

COFACTOR REQUIREMENTS FOR OXIDATION OF PYRUVATE

SYSTEM	OXYGEN UPTAKE IN μATOMS	ORTHOPHOSPHATE ESTERIFIED IN μMOLES	P/O
Complete	2.7	5.6	2.1
+ yeast coenzyme	5.3	12.3	2.3
+ yeast coenzyme but without malate	0.6	1.2	2.0
+ DPN	3.6	9.6	2.7
+ CoA	4.0	9.7	2.4
+ DPN and CoA	4.5	12.3	2.7

The complete system contained the components shown in table I without yeast coenzyme concentrate but with 20 μmoles potassium phosphate. The substrate consisted of 20 μmoles potassium pyruvate and 2 μmoles sodium L-malate. Where indicated, 0.3 μmoles DPN and 0.1 μmoles CoA were added. The reaction was stopped after 25 min.

were found to oxidize organic acids of the Krebs cycle less rapidly than those from younger leaves. Thus, in an experiment under the reaction conditions described in table I with particles from older leaves, succinate elicited the uptake of 5.7 μatoms of oxygen (P/O = 1.6), and malate 6.6 μatoms (P/O = 2.0). Glycolate, on the other hand, consumed 9.2 μatoms in the same experiment. Thus glycolate was oxidized at about twice the rate by particles from older leaves than by those from younger leaves, but succinate and malate were oxidized at half the rate. The protein N in a preparation of the particles from older leaves was not less than that ordinarily present in particles from the same weight of young leaves.

ATTEMPTS TO DEMONSTRATE OXIDATIVE PHOSPHORYLATION WITH GLYCOLATE: The P/O ratio that could be obtained consistently with glycolate as substrate was about 0.1. This represents a smaller disappearance of phosphate than can be accurately determined by the phosphomolybdate method employed, but it could be confirmed by the considerably more sensitive assay of the glucose-6-phosphate formed. A number of attempts were made to increase the phosphorylation with glycolate as substrate without success. Among the experimental conditions that were varied were: the physiological age of the spinach leaves from which the particles were prepared; the age of the particle preparations; the centrifugal force used to obtain the particles (500 × G, 10,000 × G, 100,000 × G); the kinds and amounts of coenzymes added; the substrate concentration; the presence of additional substrates; the reaction time; the concentration of orthophosphate; the addition of dialyzed or undialyzed portions of the supernatant fluid; the addition of excess catalase; the addition of semicarbazide to combine with the glyoxylate produced in the oxidation of glycolate, and increased hexokinase concentration. It must be concluded that particles capable of carrying out oxidative phosphorylation cannot do this effectively with glycolate as the substrate.

It has been suggested that glycolic acid oxidase accounts for a significant proportion of the oxygen taken up by leaves in sunlight (19, 20). The above observations indicate, however, that this oxidase cannot provide much ATP for use by the leaf.

EFFECT OF ENZYME INHIBITORS ON OXIDATION OF SUCCINATE AND GLYCOLATE: The system which oxidizes glycolate differs from that involved in the oxidation of the other substrates tested in that there is little or no associated oxidative phosphorylation (table I). Differences in the mode of oxygen uptake by glycolate and other organic acids of the Krebs cycle can also be demonstrated with suitable inhibitors. A series of competitive and highly specific inhibitors of purified glycolic acid oxidase has been described (18). These compounds are all *a*-hydroxysulfonates, and *a*-hydroxy-2-pyridinemethanesulfonic acid is the most effective of these compounds in vivo (20). As shown in experiment 1 (table III), the potassium salt of this inhibitor has no effect on succinate oxidation or on phosphorylation, but at a concentration of 5×10^{-4} M it inhibits the oxidation of glycolate to the extent of 68 %. Azide, on the other hand, inhibits the oxidation of succinate by 67 % and also inhibits phosphorylation to a marked degree. The stimulation of the oxygen uptake observed with

TABLE III

OXIDATION AND PHOSPHORYLATION WITH SUCCINATE AND GLYCOLATE IN PRESENCE OF INHIBITORS

EXPT NO.	SUBSTRATE	WITHOUT INHIBITOR	*a*-HYDROXY-2-PYRIDINEMETHANESULFONATE (5×10^{-4} M)	K CYANIDE (5×10^{-3} M)	NA AZIDE (1×10^{-3} M)
1	Succinate	10.4 (1.9)	10.1 (1.7)		3.4 (1.2)
	Glycolate	3.8	1.2		5.1
2	Succinate	9.1 (1.5)		0 (0)	
	Glycolate	5.2		6.2	

* Figures in parentheses represent P/O ratios observed.

Leaf particles and reaction mixtures were prepared and treated as described in table I. Cyanide was also placed in the center well (11) in experiments with this inhibitor. The reaction was stopped after 25 min in experiment 1, and after 20 min in experiment 2.

glycolate, on the addition of azide, occurs presumably because the catalase in the particles is inhibited and hence more oxygen is taken up per mole of glycolate oxidized (8, 21). Thus the pathway of electron transport by these particles in the presence of succinate is undoubtedly through the cytochrome system, whereas with glycolate a flavoprotein oxidase is involved. This conclusion is further borne out in experiment 2, in which cyanide was found to inhibit oxidation of succinate completely, but oxidation of glycolate was stimulated (16).

INHIBITION OF ORGANIC ACID OXIDATIONS BY GLYCOLATE AND GLYOXYLATE: Some unusual effects have been observed when certain organic acids of the Krebs cycle are oxidized by particles from spinach leaves in the presence of glycolate, or its oxidation product glyoxylate. Table IV shows that the simultaneous oxidation of glycolate and either succinate or citrate inhibits the normal oxygen uptake achieved by each acid alone. Similar mutual inhibitions have been observed by Pierpoint (14) when two substrates of the Krebs cycle were supplied to particles from tobacco leaves. This was ascribed to the saturation of some intermediate reactant in the oxidation system common to both substrates. In the oxidation of glycolate, however, where no common intermediate exists (table III), some other mechanism must obtain.

Glyoxylate is oxidized only slowly by these preparations (table IV). In spite of the inhibitions of the oxidation of succinate and citrate caused by glycolate and glyoxylate, no lowering of the P/O ratio for either of these substrates occurs.

DISCUSSION

The rates of oxidation, and the efficiency of oxidative phosphorylation in particles from spinach leaves compare favorably with the highest figures reported for particles from other plant parts (2), and for various tissues of animal origin (7). There is little doubt, therefore, that these leaves are capable of classical Krebs cycle activity and accompanying synthesis of ATP. The question of what part this scheme plays in the metabolism of the leaf is, of course, not answered by information of this kind. It should be emphasized that the isolated particles oxidize succinate at a rate (table I) found to be only 20 % of the endogenous dark respiration by a weight of intact leaf equal to that from which the particles were obtained. Nevertheless, for many studies of leaf biochemistry, the system described here is a convenient one. The plant material used is readily available, can be stored, and the activity of the particulate preparations is high and reproducible.

It has been suggested that pyruvate is oxidized in photosynthesizing tissues less rapidly in the light than in the dark (1, 6), so that differences apparently exist in the mechanism of respiration under these two conditions. Since glycolate is produced by leaves almost exclusively in the light, and has been shown in this study to inhibit the oxidation of substrates of the Krebs cycle, it is tempting to ascribe the effect of light on respiration to such a mechanism. A way in which glyoxylate may specifically inhibit the Krebs cycle is suggested by evidence that this compound and oxaloacetate may react to form an inhibitor of the oxidation of citrate (3).

SUMMARY

The isolation from green spinach leaves of particles which carry out oxidative phosphorylation with high P/O ratios is described. Conditions are given

TABLE IV

INHIBITION OF SUBSTRATE OXIDATION BY GLYCOLATE AND GLYOXYLATE

SUBSTRATE	TOTAL O_2 UPTAKE IN μATOMS	O_2 UPTAKE OF CITRATE OR SUCCINATE IN μATOMS	O_2 UPTAKE OF GLYCOLATE OR GLYOXYLATE IN μATOMS	ORTHOPHOSPHATE ESTERIFIED IN μMOLES	P/O FOR SUCCINATE OR CITRATE
Succinate		14.2		27.8	2.0
Citrate		8.1		22.4	2.8
Glycolate			5.1	0.7	0.1
Glyoxylate			0.7	0	0
Succinate + glycolate	12.8	9.9	2.9	18.9	1.9
Succinate + glyoxylate	9.6	8.9	0.7	15.6	1.7
Citrate + glycolate	7.9	5.2	2.7	14.1	2.7
Citrate + glyoxylate	6.3	5.6	0.7	14.2	2.5

Reaction mixtures were prepared as described in table I, and 5 μmoles of glyoxylate or 20 μmoles of glycolate were added where indicated. Oxygen uptake of glycolate in the presence of succinate or citrate was estimated from measurement of the glyoxylate formed based on the observed stoichiometry of the reaction: glycolate + 1/2 O_2 → glyoxylate + H_2O. The total oxygen uptake less that quantity derived from the glyoxylate determination was considered to be the oxygen consumed by citrate or succinate. Oxygen uptake given for the Krebs cycle substrates in the presence of glyoxylate represents the total oxygen consumed less that utilized in the control for the oxidation of glyoxylate alone. The reaction was stopped after 30 min.

for the oxidation of pyruvate, citrate, L$_s$-isocitrate, α-ketoglutarate, succinate, fumarate, L-malate, and reduced diphosphopyridine nucleotide. Some of the glycolate oxidizing activity of the leaf is also associated with this particulate system, but this reaction is not accompanied by significant synthesis of adenosine triphosphate.

Studies with enzyme inhibitors indicate that oxygen uptake is mediated by the cytochrome system when organic acids of the Krebs cycle are the substrates, and by a flavoprotein oxidase with glycolate. Certain inhibitory effects of glycolate and glyoxylate on the operation of the Krebs cycle have been described and their possible physiological importance considered.

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Literature Cited

1. Bassham, J. A., K. Shibata, K. Steenberg, J. Bourdon, and M. Calvin 1956. The photosynthetic cycle and respiration: light-dark transients. Jour. Amer. Chem. Soc. 78: 4120–4124.
2. Conn, E. E. and L. C. T. Young 1957. Oxidative phosphorylation by lupine mitochondria. Jour. Biol. Chem. 226: 23–32.
3. D'Abramo, F., M. Romano, and O. Ruff 1958. Inhibition of citrate oxidation by glyoxylate in rat-liver homogenates. Biochem. Jour. 68: 270–275.
4. Friedemann, T. E. and G. E. Haugen 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. Jour. Biol. Chem. 147: 415–442.
5. Frigerio, N. A. and H. A. Harbury 1958. Preparation and some properties of crystalline glycolic acid oxidase of spinach. Jour. Biol. Chem. 231: 135–157.
6. Gibbs, M. 1953. Effect of light intensity on the distribution of C^{14} in sunflower leaf metabolites during photosynthesis. Arch. Biochem. Biophys. 45: 156–160.
7. Hunter, F. E., Jr. 1955. Coupling of phosphorylation with oxidations. In: Methods in Enzymology, II. S. P. Colowick and N. O. Kaplan, eds. Pp. 610–616. Academic Press, New York
8. Kenten, R. H. and P. J. G. Mann 1952. Hydrogen peroxide formation in oxidations catalyzed by plant α-hydroxy acid oxidase. Biochem. Jour. 52: 130–134.
9. Korkes, S., A. del Campillo, I. C. Gunsalus, and S. Ochoa 1951. Enzymatic synthesis of citric acid. IV. Pyruvate as acetyl donor. Jour. Biol. Chem. 193: 721–735.
10. Kornberg, A. and B. L. Horecker 1955. Glucose-6-phosphate dehydrogenase. In: Methods in Enzymology, I. S. P. Colowick and N. O. Kaplan, eds. Pp. 323–326. Academic Press, New York
11. Krebs, H. A. 1935. Metabolism of amino acids. III. Deamination of amino acids. Biochem. Jour. 29: 1620–1644.
12. Lohmann, K. and L. Jendrassik 1926. Kolorimetrische Phosphorsäurebestimmugen im Muskelextrakt. Biochem. Zeits. 178: 419–426.
13. Ohmura, T. 1955. Oxidative phosphorylation by a particulate fraction from green leaves. Arch. Biochem. Biophys. 57: 187–194.
14. Pierpoint, W. S. 1959. Mitochondrial preparations from leaves of tobacco (*Nicotiana tabacum*). Biochem. Jour. 71: 518–528.
15. Radin, N. S. and D. E. Metzler 1955. Sodium glyoxylate monohydrate. Biochem. Preparations 4: 60–62.
16. Tolbert, N. E. and R. H. Burris 1950. Light activation of the plant enzyme which oxidizes glycolic acid. Jour. Biol. Chem. 186: 791–804.
17. Vickery, H. B. and D. G. Wilson 1958. Preparation of potassium dihydrogen L$_s$(+)-isocitrate from *Bryophyllum calycinum* leaves. Jour. Biol. Chem. 233: 14–17.
18. Zelitch, I. 1957. α-Hydroxysulfonates as inhibitors of the enzymatic oxidation of glycolic and lactic acids. Jour. Biol. Chem. 224: 251–260.
19. Zelitch, I. 1958. The role of glycolic acid oxidase in the respiration of leaves. Jour. Biol. Chem. 233: 1299–1303.
20. Zelitch, I. 1959. The relationship of glycolic acid to respiration and photosynthesis in tobacco leaves. Jour. Biol. Chem. 234: 3077–3081.
21. Zelitch, I. and S. Ochoa 1953. Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. Jour. Biol. Chem. 201: 707–718.

EFFECT OF GROWTH ON REDISTRIBUTION OF SOME MINERAL ELEMENTS IN PEPPERMINT[1, 2]

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Biddulph (1) suggests that specific mineral elements show characteristic distribution patterns within a plant. The work of Sayre (9) and Langston (6, 7) has verified this point. More recently Biddulph et al (2) re-emphasized that elements actually undergo a circulating type of movement in the plant. The degree of movement of any element in a plant, of course, varies quite widely from element to element.

It is the purpose of this study using radioactive elements and radioautographic techniques to show the effect of growth on the redistribution of the elements Ca^{45}, S^{35}, Zn^{65}, Cl^{36}, and Co^{60} in the peppermint plant.

MATERIALS AND METHODS

Peppermint (*Mentha piperita* L.) stock plants were grown in the greenhouse under a short day photoperiod to maintain the plants in a vegetative condition. Cuttings were made from the stock plants at various times as the need arose. After roots appeared, the cuttings were grown on half strength Hoagland and Arnon nutrient solution (5). At the time of use, cuttings were selected for uniformity. Only cuttings with at least three mature leaves and with a height of approximately 5 in were used. Before treatment, cuttings were removed from the rooting medium and their roots were washed free of the sand.

Twenty cuttings were used per treatment. Table I lists the various radioisotopes employed, activities, chemical form, and amount of time of uptake by the plants. In all cases the isotope was added to full strength Hoagland and Arnon nutrient solution.

TABLE I

ACTIVITIES AND CHEMICAL FORM OF RADIOISOTOPES EMPLOYED AND LENGTH OF TIME OF UPTAKE

RADIOISOTOPE	CHEMICAL FORM	ACTIVITY OF SOL.	UPTAKE TIME IN HRS
C^{45}	$CaCl_2$	3.0 μc/ml	4
S^{35}	H_2SO_4	3.4 μc/ml	4
Co^{60}	$CoCl_2$	3.0 μc/ml	4
Cl^{36}	HCl	0.33 μc/ml	4
Zn^{65}	$ZnCl_2$	3.0 μc/ml	96

At the end of the initial 4 hour uptake under daylight greenhouse conditions the cuttings were assayed, and it was found that they all had high activity except the zinc treatment. The zinc treatment cuttings were then allowed to remain in the solution for a total period of 96 hours. No attempt was made to ascertain total activity as a result of uptake.

At the end of the uptake period one plant from each treatment was pressed and dried to show initial uptake. The remaining plants had their roots removed and were again placed in rooting flats, two treatments per flat. When roots were well developed the young plants were transferred to 3 in plastic pots. At the time of potting two more plants from each treatment were pressed and dried and radioautographs prepared.

Thereafter every 2 weeks for 8 weeks two plants were removed from the pots and their roots washed free of soil. They were then pressed, dried, and radio-

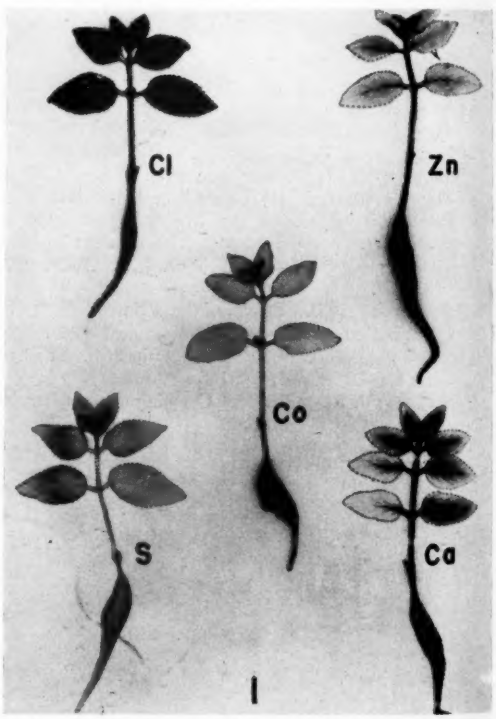

FIG. 1. Radioautographs showing initial uptake of the various radioisotopes.

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autographed. In all cases only the results obtained after 42 and 72 days growth are reported since these radioautographs represent an overall picture of the data obtained. In an effort to make the radioautographs more easily interpreted the plants have been outlined by dashed India ink lines.

The method of pressing and drying the plants was as follows. The plants were dried in a herbarium press at 75° C for 24 hours. The plants were then mounted with Duco cement on 14 by 17 in cardboard.

The radioautographs were made with no-screen X-ray film by placing the film in direct contact with the plant material in a weighted X-ray exposure holder. After exposure the films were developed in the normal manner.

Results and Discussion

These studies were designed to investigate the effect of growth on the redistribution of several isotopes of nutrient elements. Root removal after initial absorption of the isotope allowed easy studies of downward and upward movements. Further, removal of the roots eliminated the movement of any isotope absorbed on the roots, which would have confused the radioautographic results.

The initial accumulations of Calcium 45 were deposited in the stem, petiole and vein tissues of the leave (fig 1). These accumulations resulted in a characteristic distribution pattern as previously shown by Sayre (9), Biddulph (1), and Langston (6). Peppermint plants after 42 days growth show similar distribution patterns of Calcium 45 (fig 2). Redistribution of Calcium 45 in an upward direction is readily apparent. This is shown both in the young upper leaves as well as in the auxillary shoots. Further, Calcium 45 redistribution in a downward direction in the new roots was easily detected by the radioautographic technique. After 72 days growth the initial distribution pattern is maintained (fig 3). It is apparent however after this rather large quantity of growth (note lateral shoots) that those areas that initially accumulated Calcium 45 have retained the bulk of it. However, it must be pointed out that even in the meristems after 72 days some calcium is being accumulated indicating redistribution. This does not appear to be true in the case of downward redistribution as the root tips show no detectable Calcium 45.

Mason and Maskell (8) showed that calcium does not move in the phloem tissue of the cotton plant. Biddulph et al (2) indicated that the downward path of mineral element movement within a plant is in the phloem tissue. It appears that in peppermint a limited quantity of calcium is redistributed in a downward direction as a result of growth. In contrast to the limited downward redistribution, relatively large quantities move into apical areas and into lateral

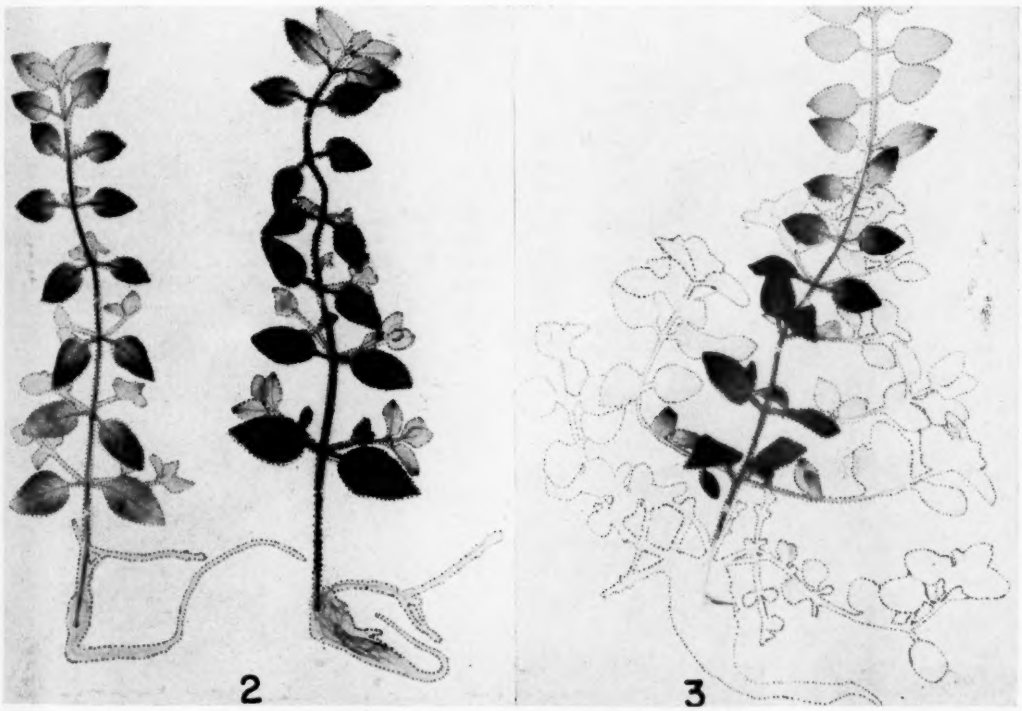
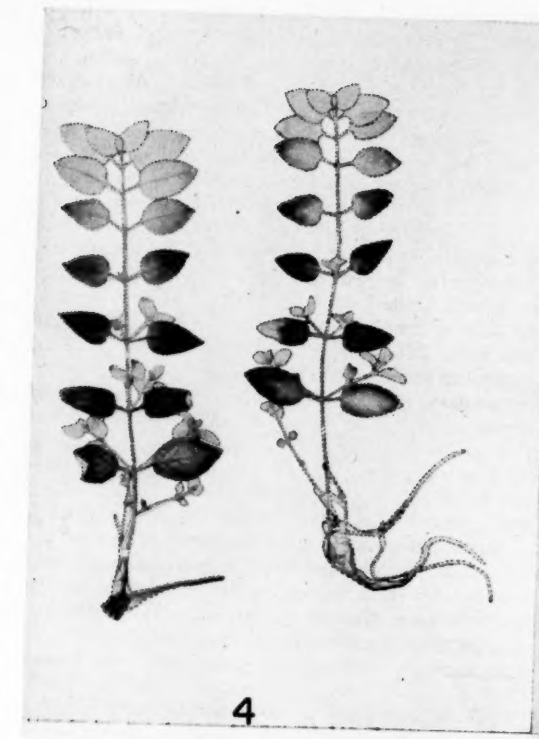


Fig. 2. Radioautograph showing the effect of 42 days growth on the redistribution of Ca^{45}.
Fig. 3. Radioautograph showing the effect of 72 days growth on the redistribution of Ca^{45}.



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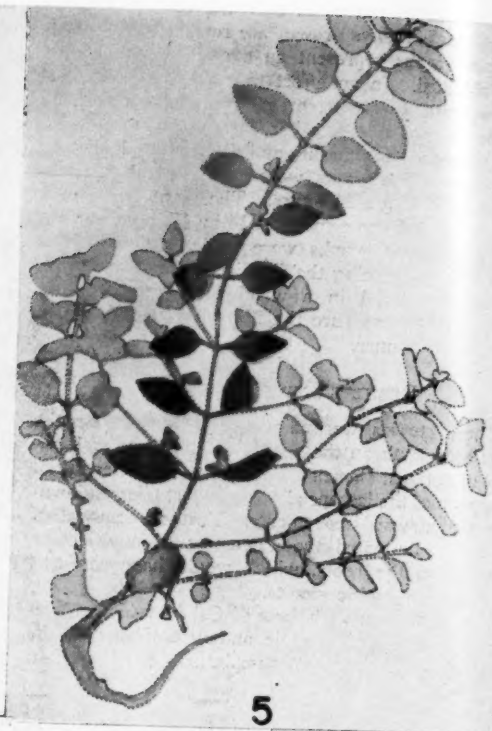

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shoot growth. This is not in complete agreement with the work of the previously mentioned research (1, 2, 8), but agrees with that of Ferrell (4).

If the radioautographs are observed critically they show that the calcium which was redistributed upwardly came from the stem tissue and not the leaf tissue (figs 2 & 3). Accumulations in the leaves remain constant while growth reduces that in the stem tissue. Thus, by the technique used it appears the calcium in the stem tissue is more readily mobile than that in the leaf tissue.

There was relatively more sulphur in the mesophyll region of the S^{35} treated plant than there was calcium in the same region in the Ca^{45} treated plant (fig 1). After 42 days of growth, (fig 4) the sulphur shows a more even redistribution in the mesophyll region of the leaves. Note that the developing lateral shoots and the developing stolons contain a rather uniform distribution of sulphur. In the newly developed roots sulphur redistribution can also be observed.

After 72 days of growth the typical sulphur distribution pattern observed initially still remains (fig 5). The new apical, lateral, and root growth shows redistribution quite well. At this time a granular precipitate noted after 42 days of growth is even more pronounced. It is quite clear in the mesophyll region of the old leaf tissue and in the veinal and stem tissue of the new growth. In one area it can be observed that the precipitation is taking place in the vascular tissue of the new lateral stem growth. This effect was even much more striking in the original radioautographs.

The results observed with sulphur are in agreement with the conclusions of Biddulph et al (3) and Thomas et al (11). It seems quite apparent that part of the radioactive sulphur absorbed by the treated cuttings has remained mobile within the plant and has been retranslocated throughout the entire plant as growth continued. This can be seen by observing the new growth on the plants. The appearance of radioactive sulphur in the new roots means that some of the sulphur absorbed by the plant has undergone a true circulating type of movement within the plant. There does not seem to be any loss of sulphur from the older mature leaves of the plant indicating that the sulphur which was redistributed throughout the plant did not come from these areas. It would appear, therefore, that some of the sulphur remained in a mobile condition in the stem tissues.

Further, as the plants grew, the distribution of sulphur became more uniform in the leaf vein and leaf mesophyll tissue. In some instances it can be noted that the mesophyll region has a greater accumulation than the veinal region of the leaf as shown by Langston (6), Biddulph (1), Biddulph et al (2), and Sayre (9).

The initial distribution pattern of Zn^{65} is maintained throughout the length of the study (figs 1, 6, and 7). There is a lack of accumulation in the mesophyll region of the leaf tissue. The plants harvested at 42 and 72 days showed very marked upward and downward redistribution of Zn^{65}. As can be expected the distribution pattern remains the same even after large quantities of growth. In addition to the transport area, heavy accumulations of zinc occur at the nodal areas of the stolon and lateral shoot growth.

It is apparent that the zinc is redistributed throughout the areas of new growth. Redistribution seems to be more in an upward direction than in a downward direction with a heavier concentration appearing in the new leaf growth than in the new root growth. The older mature leaves do not show a decrease in zinc concentration as also noted by Wood and Sibly (13) and Shaw et al (10). Thus, zinc showed a pattern somewhat similar to sulphur in that some zinc has remained in a mobile condition throughout the plant; this zinc is readily redistributed to areas of new growth. The appearance of zinc in the newly formed roots indicates that some of the zinc has undergone a circulation type of movement in the plants.

Chlorine distribution in peppermint is shown in figures 1, 8, and 9. After 42 days growth (fig 8) redistribution of chlorine is noted in the stem tissue of the developing lateral buds and as growth progressed, larger quantities were distributed in a downward direction with heavy accumulations appearing in the lower lateral shoots and roots.

After 72 days growth (fig 9) the redistributed chlorine came from the older mature leaves, since there was a decrease in concentration of chlorine in the older leaves of plants harvested after 42 days, as compared to those harvested after 72 days. This redistribution seems to be in a predominately downward direction. Radioautographs made between 42 and 72 days growth and not shown in this report indicate that this downward movement progressed from leaf pair to leaf pair. This is partially borne out by figure 9. There is a heavy accumulation of chlorine in the stem, petiole, and vein tissue.

Chlorine is redistributed throughout the plant as reported by Woolley et al (14). An interesting observation to note is the noticeable downward redistribution of the element chlorine as growth progressed. This is in agreement with the work of Ulrich and Ohki (12) who demonstrated that one of the deficiency symptoms of chlorine is stubby root growth. Chlorine has undergone a circulation type of movement.

FIG. 4. Radioautograph showing the effect of 42 days growth on the redistribution of S^{35}.
FIG. 5. Radioautograph showing the effect of 72 days growth on the redistribution of S^{35}.
FIG. 6. Radioautograph showing the effect of 42 days growth on the redistribution of Zn^{65}.
FIG. 7. Radioautograph showing the effect of 72 days growth on the redistribution of Zn^{65}.

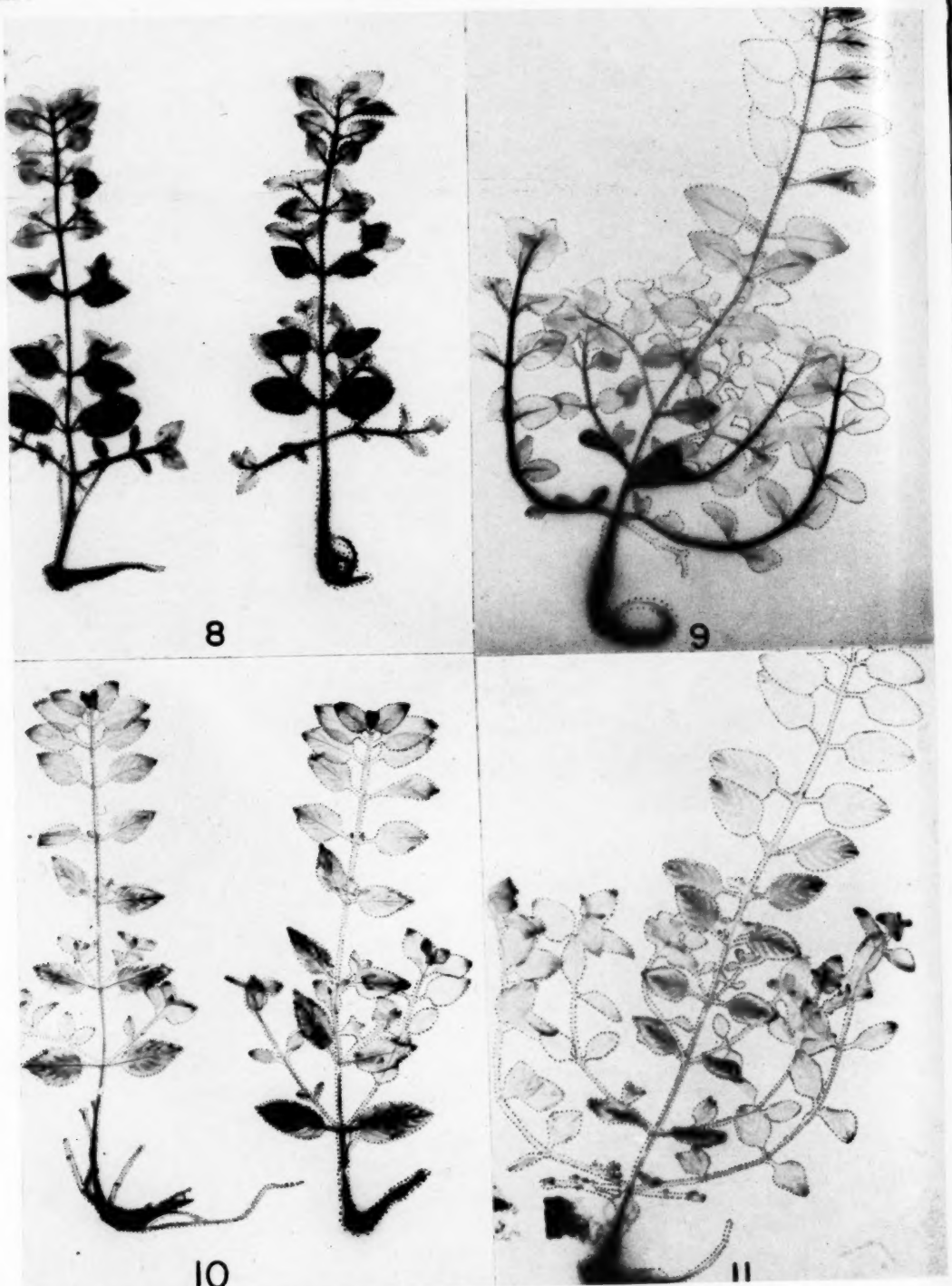

FIG. 8. Radioautograph showing the effect of 42 days growth on the redistribution of Cl^{36}.
FIG. 9. Radioautograph showing the effect of 72 days growth on the redistribution of Cl^{36}.
FIG. 10. Radioautograph showing the effect of 42 days growth on the redistribution of Co^{60}.
FIG. 11. Radioautograph showing the effect of 72 days growth on the redistribution of Co^{60}.

The general distribution pattern of cobalt can be seen in figures 1, 10, and 11. The major region of cobalt accumulation is at the leaf tips and leaf margins. This typical distribution pattern was maintained by redistribution throughout the growth of the plants during the 72 days of the experiment. In fact, the accumulation at the leaf tips and leaf margins is much more pronounced as the plants grow older, also there is a large redistribution of the cobalt to the new root tissue.

There seems to be no other definite area of cobalt accumulation. Cobalt remains entirely mobile within the plant, showing up in all the areas of new growth; like chlorine, relatively large quantities of cobalt have been retranslocated to the roots showing a circulation type of movement.

Summary

Radioautographic techniques were used to determine the long term distribution patterns of C^{45}, S^{35}, Zn^{65}, Cl^{36}, and Co^{60}. Each element maintained a specific distribution pattern throughout the experiment. The type of feeding technique used enabled one to follow both the upward and downward movement of the element in question over a long period of time.

The calcium in the older mature leaves is immobile while some of the calcium remains free in the transport tissues and is able to be redistributed in an upward direction. Calcium is only very slightly mobile in a downward direction in the plant.

Sulphur is accumulated more evenly in the mesophyll region of the leaves. Also, sulphur in the older mature leaves seems immobile while the free sulphur within the transport tissues is mobile in both an upward and downward direction.

Zinc accumulates primarily in stem tissue, petiole tissue, and vein tissue. The redistribution of zinc occurs throughout the plants studied; it appears to come from the free zinc within the plant. The zinc in the older mature leaves remained immobile.

Chlorine was shown to have a definite downward redistribution as growth proceeds. Unlike the other elements studied the redistributed chlorine came from older leaves.

Areas of cobalt accumulation are the margins and tips of the leaves. No other areas seem to act as definite areas of accumulation. Cobalt is completely mobile within the plant.

The elements sulphur, zinc, chlorine, and cobalt have all shown circulation types of movement while this type of movement is questionable with regard to calcium.

Literature Cited

1. Biddulph, O. 1953. Translocation of Radioactive Mineral Nutrients in Plants. Use of Isotopes in Plant and Animal Research. Pp. 48–58. U. S. Atomic Energy Com., TID–5098.
2. Biddulph, O., S. Biddulph, R. Cory, and H. Koontz 1958. Circulation patterns for phosphorus, sulfur and calcium in the bean plant. Plant Physiol. 33: 293–300.
3. Biddulph, O., R. Cory, and S. Biddulph 1956. The absorption and translocation of sulfur in red kidney bean. Plant Physiol. 31: 28–33.
4. Ferrell, W. K. 1956. Mobility of calcium 45 after injection into western white pine. Science 124: 364–365.
5. Hoagland, D. R. and D. I. Arnon 1938. The Water-Culture Method for Growing Plants Without Soil. University Calif. Agr. Exp. Sta. Circ. 347.
6. Langston, R. 1956. Distribution patterns of radioisotopes in plants. Proc. Amer. Soc. Hort. Sci. 68: 370–376.
7. Langston, R. 1956. Studies on marginal movement of cobalt 60 in cabbage. Proc. Amer. Soc. Hort. Sci. 68: 366–369.
8. Mason, T. G., and E. J. Maskell 1936. Further studies on transport in the cotton plant. I. Preliminary observations on the transport of P, K, and Ca. Ann. Bot. 45: 125–173.
9. Sayre, J. D. 1952. Accumulation of Radioisotopes in Corn Leaves. Ohio Agr. Exp. Sta. Res. Bul. 723.
10. Shaw, E., R. G. Menzel, and T. A. Dean 1954. Plant uptake of zinc 65 from soils and fertilizers in the greenhouse. Soil Sci. 77: 205–214.
11. Thomas, M. D., R. H. Hendricks, L. C. Bryner, and G. R. Hill 1944. A study of the sulphur metabolism of wheat, barley, and corn using radioactive sulphur. Plant Physiol. 19: 227–244.
12. Ulrich, A., and K. Ohki 1956. Chlorine, bromine, and sodium as nutrients for sugar beet plants. Plant Physiol. 31: 171–181.
13. Wood, J. G. and P. M. Sibly 1950. The distribution of zinc in oat plants. Australian Jour. Sci. Research SCR (B) 3: 14–27.
14. Woolley, J. T., T. C. Broyer, and G. V. Johnson 1958. Movement of chlorine within the Plant. Plant Physiol. 33: 1–7.

STUDIES ON LATERAL MOVEMENT OF PHOSPHORUS 32 IN PEPPERMINT[1,2]

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Auchter (1) was the first to show that very little lateral movement of nutrient elements occurred in certain plants. The work of Caldwell (3) and McMurtrey (7) also indicated a lack of lateral movement of mineral elements in the test plants used. From these studies, it seems that there is a lack of lateral movement of mineral nutrients in some plants. It was decided to test this idea using the radioactive element P^{32}, in peppermint (*Mentha piperita* L.) plants.

The purpose of this study was to investigate, using a split root technique and P^{32}, the type of movement that occurs when a peppermint plant is fed through a specific portion of its root system.

MATERIALS AND METHODS

The square stem of peppermint, when allowed to root, characteristically develops roots at each of the four corners of the stem allowing a rather ideal separation of the four main vascular bundles.

Peppermint stock plants were grown in the greenhouse under a short day photoperiod to maintain the plants in a vegetative condition. Cuttings were made from the stock plants at various times as the need arose. After rooting, the cuttings were then placed on full strength nutrient solution (5) until they were used. Each plant used contained at least seven pairs of mature leaves.

In the method used to feed just half the root system, the basal portion of the stem was slit longitudinally 3 to 3½ in with a razor blade. This di-

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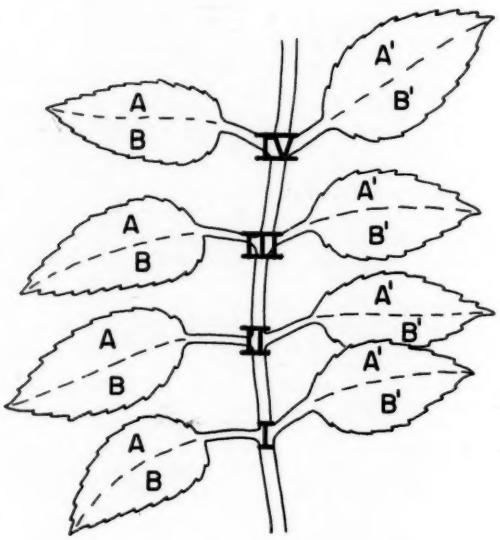
[2] Journal Paper No. 1460. Purdue University Agri. Expt. Sta., Lafayette, Ind.



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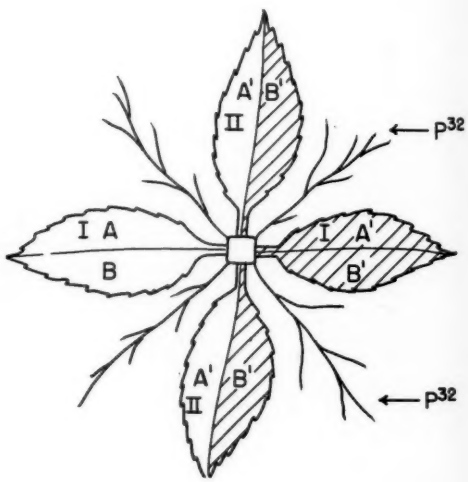

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FIG. 1. Diagram of a peppermint plant showing the symbols designating the different areas as related to the radioautographs which were used in studying the lateral movement of phosphorus.

FIG. 2. Diagram of a peppermint plant showing results of one-half feeding techniques as seen in figure 3. Leaves III, V, etc. in figure 3 are above I. Leaves IV, VI, etc. in figure 3 are above II.

vided the root system into two equal parts. When just a quarter of the plant was to be fed, one of the half sections was then split in half.

The feeding apparatus consisted of plastic Crown Freez-Tainers. These containers held 405 ml of nutrient solution up to the fill line. Three containers were lined up next to each other and held in place by means of Scotch tape. Three rows of such containers were used per feeding apparatus making a total of nine containers. Bamboo stakes supported the plants.

Plants were placed in the containers in such a manner that one-half or one-fourth of the roots, depending upon the type of split, were placed in center container. The remaining roots of the plant would then be placed in one of the two adjacent containers. The containers were large enough to allow four plants to be placed next to each other which gives a total of eight plants per three containers.

After the plants were placed in the containers, the containers were covered with aluminum foil. One week was allowed to pass before feeding the plants radioactive phosphorus. This was to allow healing of the wound caused by the splitting and also to allow new roots to form.

At the time of feeding, the containers were filled to the fill line indicated on the containers and 50 ml of nutrient solution was pipetted out of the center container. This was immediately replaced with 50 ml of radioactive phosphorus at a strength of 5.5 μc/ml. Four plants were harvested at 6, 24, 48, 96, and 144 hours after applying P^{32}. Each harvest period represented four plants which were fed P^{32} through one half or one fourth of its root system.

The method of pressing and drying the plants was as follows. The plants were dried in a herbarium press at 75° C for 24 hours. The plants were then mounted with Duco cement on 14 by 17 in cardboard. The radioautographs were made with no-screen X-ray film by placing the film in direct contact with the plant material in a weighted X-ray exposure holder. After exposure the films were developed in the normal manner.

Results

To be able to interpret the results of this study, one must first examine figure 1 and become acquainted with the marking procedure of the leaf pairs and the individual leaves. Leaves of peppermint have an opposite decussate phylotaxis with a phylotactic fraction of one-third.

Leaf pairs are designated by Roman numerals. The lowest pair of leaves being pair I, the next pair of leaves pair II, and so forth up to the stem. The right leaf of a pair of leaves as seen when one is observing a mint plant directly in front of him is designated by a prime (′) sign. The individual leaves of a pair of leaves are further subdivided into sections A and B, section A being the farthest half of an individual leaf as one is observing the plant when it is directly in front of him. Section B then is the closest half of the individual leaf.

Once the plants are pressed, the leaves all seem in the same plane but this is not how they grow. In both the radioautographs the odd numbered pairs of leaves (pairs I, III, V, etc.) were growing as they appear in the radioautographs. The even numbered pairs of leaves (pairs II, IV, VI, etc.) were pressed in such a way that they appear 90° out of their normal position. In all cases the leaves designated by the prime (′) sign (right half) will appear to the right in the radioautographs. The other half of the pair therefore appears to the left in the radioautographs.

The results of the uptake by half the root system are shown schematically in figure 2. Figure 3 shows quite clearly that in feeding radioactive phosphorus to just half the plant's root system that the radioactive phosphorus remained essentially in just half of the plant (compare the A′ and B′ sections of the odd numbered leaves with the A and B sections). In every case where the P^{32} was fed to half the roots, there appears to be very little lateral movement to the other half of the plant.

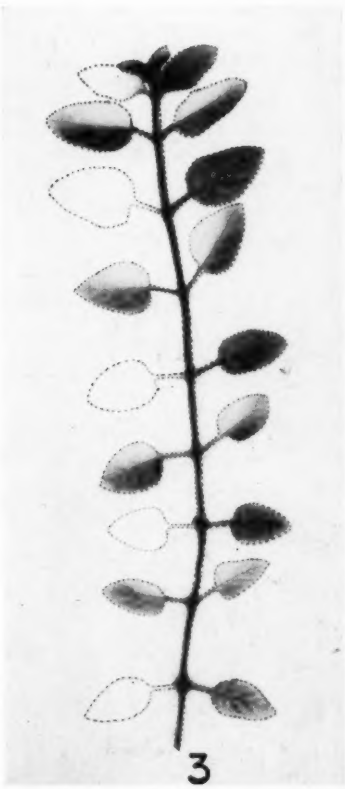

FIG. 3. Radioautograph showing distribution of phosphorus 32 in the aerial portion of the peppermint plant after feeding one-half of its roots system for a period of 144 hours.

It is also interesting to note that in the even numbered pairs of leaves (II, IV, etc.) the radioactive phosphorus is distributed in just half the individual leaf (B′ section of leaves II, IV, etc.). There is a slight movement from the B′ section to the A′ section in the even numbered leaves though it appears that this movement from one section of the leaf to the other section of the leaf is quite slow since after 144 hours feeding an even distribution of radioactive phosphorus is not noticeable in the entire leaf.

These results show that when P^{32} is fed to half of the root system of a peppermint plant that the phosphorus shows very little lateral movement to the other half of the plant.

The results of the uptake by one-fourth of the root system are shown schematically in figure 4. These results shown in figure 5 agree quite nicely with the results of the one-half root feeding technique. In feeding just one of the four main vascular bundles of the peppermint plant the radioactive phosphorus accumulates in either the A section of the leaf or the B section of the leaf depending upon which one of the vascular bundles is fed. In every case, if the radioactive phosphorus appears in the A section of the odd numbered leaves, it then appears in the B section of the even numbered leaves. The reverse of this pattern is true also. Here, too, there appears to be movement of the radioactive phosphorus from one section of a leaf to the other section. The movement here is quite slow also, as can be seen in figure 5.

These results also indicate that there is very little lateral movement of the phosphorus. These results show more clearly that the individual vascular bundles of the mint plant feed certain leaves and even a certain portion of the individual leaf involved.

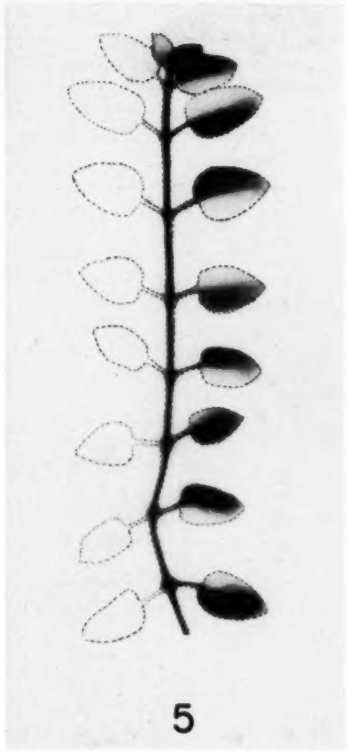

FIG. 5. Radioautograph showing distribution of phosphorus *32* in the aerial portion of the peppermint plant after feeding one-quarter of its root system for a period of 144 hours.

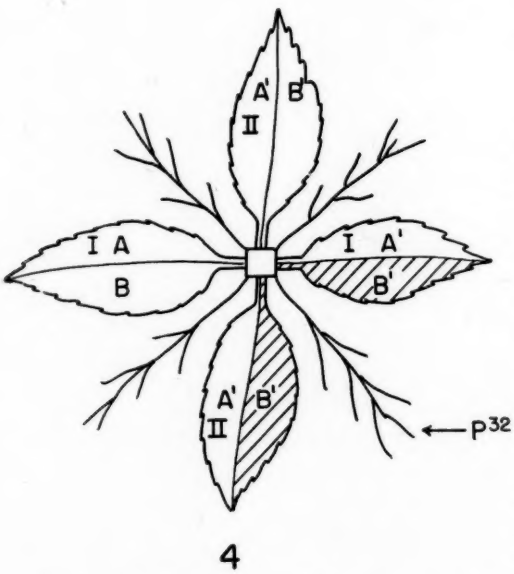

FIG. 4. Diagram of a peppermint plant showing results of one quarter feeding technique as seen in figure 5. Leaves III, V, Etc. in figure 5 are above II.

DISCUSSION

The peppermint plant was chosen for this lateral movement study because of its anatomical and morphological characteristics. *Mentha piperita* L. contains its complex vascular tissue in the four corners of the stem as shown by Chamberlin (4). She pointed out that the vascular tissues in the four corners are connected to one another by continuous interfascicular cambium which produces connecting xylem and phloem tissue. Thus, the tissues in peppermint are present in which lateral transport of minerals may take place.

The results noted with peppermint agree with the previous work done by Auchter (1), Bodenberg (2), MacDaniels and Curtis (6), Caldwell (3), and McMurtrey (7). The results show that when P^{32} is taken up by a specific portion of roots it is translo-

cated to a specific portion of the plant involved. This is shown in the radioautographs presented. The radioautographs show very little movement of the P^{32} rom one side of the peppermint plant to the other de of the plant over a period of 144 hours. The relationship between the roots feeding a specific portion of the plant is brought out even more strongly by ing the even numbered leaves of the one-half feed- technique and the leaves containing the radio- ive phosphorus in the one-quarter feeding tech- ique. In these instances the radioactive phosphorus initially was distributed in just half of an individual leaf, though some movement did occur after the initial distribution. These results agree with the work of McMurtrey (7). This, though, is not the case in strawberries as reported by White (9), and the work Ririe and Toth (8) with tomatoes.

Summary

Radioautographic techniques were used to follow feeding of radioactive phosphorus in peppermint The radioactive phosphorus was fed in such ier to feed one-half and one-quarter of the roots at a time. This type of feeding technique es one to follow lateral movement of the radio- ive tracer that is fed to the plants.

In all cases it appears quite clearly that very little lateral movement of the radioactive phosphorus took place up to 144 hours.

Literature Cited

1. Auchter, E. C. 1923. Is there normally a cross transfer of foods, water and mineral nutrients in woody plants? Md. Agri. Expt. Sta. Bul. 257.
2. Bodenberg, E. T. 1929. Lateral transfer of lithium nitrate in salix. Amer. Jour. Bot. 16: 229–237.
3. Caldwell, J. 1930. Studies in translocation. New Phytologist. 29: 27–43.
4. Chamberlin, L. 1950. A study of the vegetative anatomy of *Mentha piperita* L. M. S. thesis, Purdue University.
5. Hoagland, D. R. and D. I. Arnon 1938. The water-culture method of growing plants without soil. Univ. Cal. Agri. Expt. Sta. Circ. 347.
6. MacDaniels, L. H. and O. F. Curtis 1930. The effect of spiral ringing on solute translocation and the structure of the regenerated tissue of the apple. Cornell Agri. Expt. Sta. Mem. 133.
7. McMurtrey, J. E., Jr. 1937. Cross transfer of mineral nutrients in the tobacco plant. Jour. Agr. Research. 55: 475–482.
8. Ririe, D. and S. J. Toth 1952. Plant studies with radioactive calcium. Soil Sci. 73: 1–10.
9. White, P. R. 1927. Studies of the physiological anatomy of the strawberry. Jour. Agr. Research. 35: 481–492.

STUDIES ON 3-INDOLEACETIC ACID METABOLISM. V. EFFECT OF CALCIUM IONS ON 3-INDOLEACETIC ACID UPTAKE AND METABOLISM BY PEA ROOTS[1, 2]

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It has previously been shown that indoleacetic acid (IAA) applied to pea roots is in part degraded and in part conjugated with aspartic acid to form indoleacetylaspartic acid (IAAspA) (2, 6). While other workers (9) have reported that IAA applied to pea roots is metabolically bound to tissue protein to give a Salkowski reactive derivative, we have found no evidence of such a complex, almost all the Salkowski material in the tissues being accountable for in our studies as IAAspA or IAA itself (3). During the present studies on IAA metabolism by roots, it was found that whereas tips of intact roots accumulated IAAspA at a rate considerably greater than epicotyl segments, excised root tips almost entirely lost this ability. This decreased accumulation of IAAspA was paralleled by a decreased loss of IAA from solution. The addition of Ca^{++} or certain divalent ions in combination with sucrose completely restored the ability of roots to accumulate IAAspA but had no effect on this process in epicotyls.

MATERIALS AND METHODS

I. PLANT MATERIALS. A. *Roots.* Alaska peas were soaked for 4 hours in water and grown for 2 days in moist vermiculite in the dark at 26° C. At the end of this period the roots had reached a length of about 23 mm. Unless otherwise stated, the 5 mm tips were cut with a razor blade and 12 tips transferred to 20 ml solution in 125 ml Erlenmeyer flasks. The solutions contained 5×10^{-3} M potassium dihydrogen phosphate at pH 5.2 and various other additions as described in the text. The solutions were previously sterilized by closing the Erlenmeyers with cotton plugs and autoclaving at 20 lbs for 20 minutes. Antibiotics were added with the tissues to a concentration of 30 ppm streptomycin and 15 ppm penicillin. The same results were obtained without the addition of antibiotics, and contamination by microorganisms could be suppressed if sterile seedling roots were used. In the latter procedure the seeds were surface sterilized by immersion in 0.5 % mercuric chloride and 1 : 500 merthiolate, each for 30 minutes. The seeds were then washed and soaked in sterile water for 4 hours and transferred to autoclaved Petri dishes containing a layer of moist cellocotton. After the roots had reached a length of about 23 mm the roots were excised aseptically and transferred to the solution as above. In the experiments described the simpler procedure, using antibiotics, was used throughout.

The solutions were continuously shaken (50 complete strokes per min) at constant temperature of 25° C in the dark.

B. *Epicotyls.* Epicotyls of 7-day old seedlings were grown and incubated as previously described (2).

II. CHEMICAL ANALYSIS. A. *Determination of IAA in solution.* IAA was determined colorimetrically by the Salkowski reagent as previously described (2).

B. *Determination of IAA and IAAspA in the tissues.* At the end of the incubation period, the tissues were washed briefly in running distilled water, and then analyzed by either of two methods: 1. The tissues were transferred to 5 ml of 0.1 *N* $NaHCO_3$ solution, ground in a Potter-Elveljem microgrinder, and the brei treated as previously described, viz. by protein precipitation with ammonium sulfate followed by ether extraction of the ammonium sulfate solution at pH 4.5 and 2.5. The IAA was extracted at the higher pH, the IAAspA at the lower pH. These ether extracts were then used for colorimetric and chromatographic analysis as described (2). 2. The tissues were transferred to 5 ml of 95 % ethanol and ground. The brei was centrifuged, the supernatant poured into a beaker. The precipitate was washed with ethanol and the supernatant and washing combined. One drop of strong ammonium hydroxide was added and the solution evaporated on a steam bath in a stream of air. For chromatographic analysis, a small amount of ethanol was added, and the solution applied to paper and chromatographed as previously described (2). If only IAA or IAAspA was present in these extracts, colorimetric analysis could be carried out on the ethanol extracts, after evaporation, by adding 1 ml of 0.1 *N* $NaHCO_3$ solution and 4 ml of Salkowski reagent and determining the color photometrically as described (2). (In some experiments, the tissue precipitate was washed with $NaHCO_3$ which was then added to the ethanol evaporate; this step was found to be unnecessary.)

If IAA and IAAspA were both present in root tissues, method 1 was used for colorimetric analysis.

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[2] Contribution 155, Pesticide Research Institute, Research Branch, Canada Agriculture, University Sub Post Office, London, Ontario.

With epicotyls, unless small amounts of tissues were used, as in the first experiment, the ethanol extracts of method 2 were too turbid for either colorimetric or chromatographic analysis and only method 1 was applicable.

The results of the experiments on IAA uptake and IAAspA accumulation were expressed as μM per gram fresh weight. The fresh weights were obtained by weighing the tissues before the experiments. The initial weights of lots of twelve 5 mm tips weighed 65.1 ± 3.9 mg.

Results and Discussion

I. Intact Seedlings. The roots of twelve 2-day-old seedlings were immersed in 100 ml of 10^{-4} M IAA solution for 24 hours. Figure 1 shows the chromatograms of the entire ethanol extracts of roots (644 mg) and epicotyls (653 mg).

The chromatograms of roots show the presence of one major and several minor Salkowski reactive compounds. The major spot corresponds to IAAspA by criteria applied in our previous studies on epicotyls (2). A trace of IAA can also be found in these extracts of entire roots. Other Salkowski reactive compounds, similar in their chemical behaviour to those previously found in IAA treated epicotyls (2) also occur, but in small quantities. The extracts used in these chromatograms were obtained from about five to ten times the amount of root tissues as those used in the subsequent root studies; with the smaller amount of roots the only Salkowski reactive spot detectable on the chromatograms was IAAspA. Ether extraction of the roots gave essentially the same results. Untreated roots gave no spot at the Rf of IAAspA or IAA.

The chromatograms of the epicotyl extracts showed no distinct indole-reactive spots indicating that neither IAA nor IAAspA is transported from the treated roots to the epicotyls in detectable quantities.

If, after incubation in IAA solution, the seedlings were placed in water, the IAAspA content of the roots persisted for at least 24 hours; only after prolonged washing in water was there any decline in the IAAspA content.

II. Metabolic Activity of Various Root Zones. To assay the various root zones for their ability to conjugate IAA with aspartic acid, whole roots or root sections of twelve seedlings were incubated in solutions containing 10^{-4} M IAA for 24 hours. Three zones were analyzed for IAAspA content: the 5 mm tip, the 10 mm middle zone, and the basal portion which was almost 8 mm long. Table I shows the results with A, intact seedling roots, segmented after incubation, B. excised whole roots, segmented after a similar incubation period, and C. roots segmented before incubation. It can be seen that roots of intact seedlings and excised roots were equally active, the greatest accumulation occurring in the tips in both cases. The tips which were excised before incubation, however, almost entirely lost their ability to accumulate IAAspA, whereas the basal section was relatively unaffected. These results suggested that some factor, essential for IAAspA accumulation, becomes limiting in the metabolism of excised tips. Aqueous cotyledon extracts and various nutrient factors were therefore added to the incubation medium to determine if they could restore the ability of root tips to accumulate IAAspA.

Table I

Ability of Various Root Zones to Accumulate Indoleacetylaspartic Acid During 24 Hours

Treatments	Indoleacetylaspartic acid micromoles / gram / 24 hours — Root zones		
	1st cm 5 mm	2nd mm 10 mm	3rd about 8 mm
	1.70	0.87	0.31
	1.70	1.10	0.83
	0.22	0.36	0.60

To prepare the cotyledon extract, cotyledons of 2-day-old seedlings were ground in water, centrifuged, the supernatant adjusted to pH 4, centrifuged again, and the supernatant boiled, cooled, and centrifuged once more. The clear supernatant, corresponding to 1 % of the initial cotyledon weight, was used. As the source of nutrient factors, a synthetic medium evolved by Bonner and Addicott (4) and modified by Torrey (12) for pea root tip culture was used but without the agar. This medium contained minerals, vitamins and 4 % sucrose.

Twelve root tips were incubated in solutions containing 10^{-4} M IAA and various additions. Figure 2 shows that while cotyledon juice tripled the amounts of IAAspA found in the tips, the complete synthetic medium resulted in about a 10-fold increase. By process of elimination, it was found that 10^{-3} M $Ca(NO_3)_2$ and 0.5 % sucrose combined gave results as good as the complete medium. Sucrose alone had relatively little effect, while $Ca(NO_3)_2$ alone increased the accumulation 6-fold. The Ca^{++} effect was noted in root tips incubated either in water or phosphate buffer. Adding either Ca^{++} or sucrose alone to the cotyledon extract had no additive effect. Further studies indicated that other sugars, such as glucose or fructose, could replace sucrose, and furthermore, that sugar alcohols, such as mannitol (fig 2) or dulcitol were as effective as sucrose at the same concentration level.

Ca^{++} could be replaced by a number of divalent cations, but not by mono- or poly-valent cations.

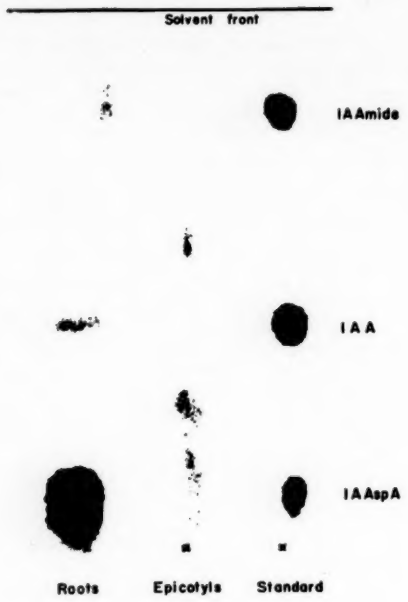

Fig. 1

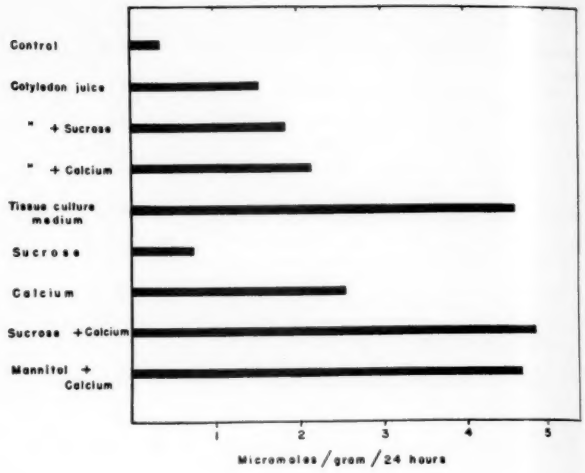

Fig. 2

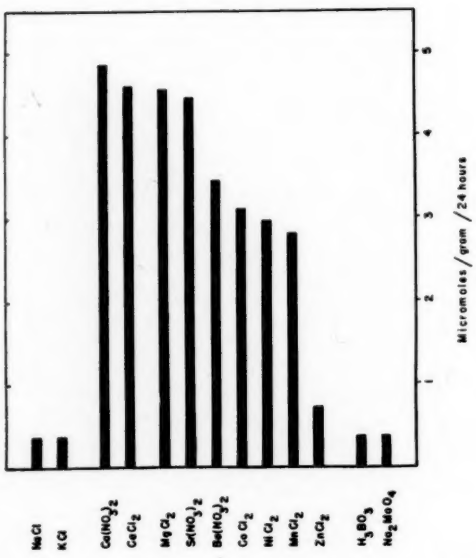

Fig. 3

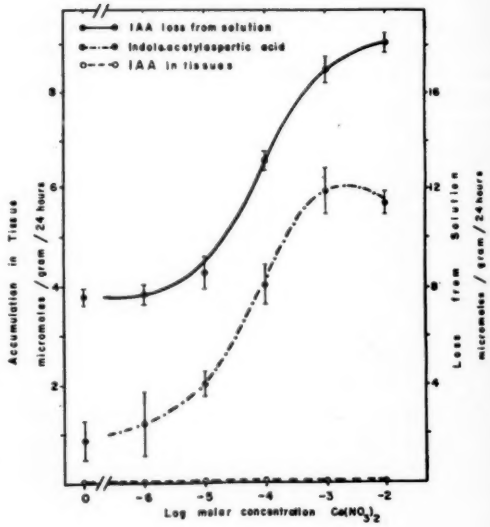
IAA loss from solution
Indoleacetylaspartic acid
IAA in tissues
Accumulation in Tissue micromoles / gram / 24 hours
Loss from Solution micromoles / gram / 24 hours
Log molar concentration Ca(NO3)2

Fig. 4

FIG. 1. Chromotograms of ethanol extracts of twelve 2-day old pea seedlings treated with 10^{-4} M IAA for 24 hours. The entire extracts of roots and epicotyls were applied. A standard solution containing indoleacetamide (IA Amide), indoleacetic acid (IAA), and indoleacetylaspartic acid (IAAspA) was also applied for reference. The chromatograms were sprayed with Salkowski reagent.

FIG. 2. Accumulation of indoleacetylaspartic acid in pea root tips incubated for 24 hours in 10^{-4} M IAA, as affected by the addition of various substances. (see text).

TABLE II

EFFECT OF AERATION OR CALCIUM ION CONTENT ON IAA LOSS FROM SOLUTION AND INDOLEACETYLASPARTIC ACID ACCUMULATION BY EXCISED ROOT TIPS

	IAA loss from solution % of control	IAAspA accumulation % of control
I. Tissues immersed*		
plus Ca – shaken	100	100
" " – anerobic under N_2	0	0
" " – no shaking	4	0
no Ca – shaken	45	9
II. Tissues breaking surface**		
plus Ca	...	38
no Ca	...	35

Twelve root tips incubated for 24 hours in 10^{-4} M IAA, 5 x 10^{-3} M potassium phosphate at pH 5.2, 0.5 % sucrose with or without 10^{-3} M $Ca(NO_3)_2$.

* Tissues completely immersed in 20 ml in 125 ml Erlenmeyer flasks.

** Tissues just breaking surface in 10 ml solution in large 15 cm diameter Petri dish.

Figure 3 shows that Mg^{++} and Sr^{++} are as active as Ca^{++}, while Ba^{++}, Co^{++}, Ni^{++}, Mn^{++} are less active and Zn^{++} almost inactive. This order is not changed if concentrations greater or less than 10^{-3} M were applied. In other experiments ethylenediaminetetraacetic acid, at equal molar concentration, completely reversed the Ca^{++} effect. Monovalent cations in combination with Ca^{++} did not alter the action of Ca^{++} even if their concentration was 10-fold that of Ca^{++}. The nature of the anion appeared immaterial as $Ca(NO_3)_2$ gave essentially the same results as $CaCl_2$ (fig 3).

Ca^{++}, to be effective, must be continuously present in the medium, for seeds which had been soaked in $Ca(NO_3)_2$ and watered with $Ca(NO_3)_2$ during the 2 days of germination, yielded roots which were as sensitive to added Ca^{++} as were roots of control seedlings treated with distilled water during their entire development.

The Ca^{++} effect could be observed only with root tips which were submerged in solution and not with root tips which were incubated in solutions so shallow that the roots broke the surface, or in tissues suspended on nylon netting on the surface of the solution. This can be seen from the results in table II which shows that the addition of Ca^{++} to the medium had little effect on the accumulation of IAAspA by partly submerged roots. It should be noted that while the accumulation of IAAspA by the partly submerged roots is four times greater than that of submerged roots in a Ca^{++} free medium, it is only about a third of the accumulation found in roots submerged in a Ca^{++} containing solution.

It seems unlikely that the submerged roots suffered from an oxygen deficiency which, in some way, was relieved by the presence of Ca^{++}. The solutions were well shaken or in some cases a stream of air was passed through the solutions without affecting the necessity for Ca^{++}. Even under completely submerged conditions, the root tips were only about ten millimeters below the surface of the solution. It should be noted, however, that good aeration was essential for the uptake of IAA since even in the presence of Ca^{++} and sucrose (table II) under anaerobic conditions, IAA was not lost from solution.

The effect of Ca^{++} and sucrose on IAAspA accumulation was not confined to excised tips, but was also observed in tips of intact roots, in which case the IAAspA content was doubled (table III).

With epicotyls, Ca^{++} and sucrose had no effect on IAAspA accumulation, which is low compared to root tips (table III). It would appear that in this respect

TABLE III

EFFECT OF CALCIUM IONS ON ACCUMULATION OF INDOLEACETYLASPARTIC ACID IN EXCISED ROOT TIPS, TIPS OF INTACT ROOTS, AND EPICOTYL SEGMENTS

	INDOLEACETYLASPARTIC ACID ACCUMULATION IN TISSUES	
	10^{-3} M $Ca(NO_3)_2$ IN MEDIUM $\mu M/g$	No $Ca(NO_3)_2$ IN MEDIUM $\mu M/g$
Excised root tips	5.9	0.4
Tips of intact roots	3.9	1.7
Epicotyl segments	0.3	0.3

FIG. 3. Accumulation of indoleacetylaspartic acid in pea root tips incubated for 24 hours in 10^{-4} M IAA, 0.5 % sucrose as affected by the addition of various mono-, di- or poly-valent cations at 10^{-3} M.

FIG. 4. Effect of calcium ion concentration on the loss of IAA from solution and the accumulation of indoleacetylaspartic acid in root tips during 24 hours. Solutions contained 10^{-4} M IAA, 0.5 % sucrose and $Ca(NO_3)_2$ at concentration as indicated. The vertical lines represent twice the standard error of the mean of three experiments.

the epicotyls resemble the basal portions of the roots (table I).

III. Action of Calcium Ions. It has been known for some time that calcium ions stimulate uptake of certain anions or cations by roots. The stimulatory action of Ca^{++} on K^+ uptake was first noted by Viets (13); other workers have also found that Ca^{++} stimulates the uptake of NO_3^- (5), $SO_4^=$ (8) and Rb^+ (11).

From figure 4 it can be seen that IAA uptake, as indicated by loss from solution, is also increased by Ca^{++} ions; IAA loss from solution, if plotted against the log concentration of Ca^{++}, gives a sigmoidal curve with a sharp rise between 10^{-5} and 10^{-4} M. As the rise in loss from solution roughly parallels the increase in IAAspA accumulation and as there is no greater accumulation of free IAA with low concentration of Ca^{++}, these results suggest that the decreased accumulation of IAAspA in the absence of Ca^{++} is due to a decreased uptake of IAA. However, the possibility that the Ca^{++} effect is on the conjugation of IAA with aspartic acid cannot be completely excluded. It should be noted that the effect of Ca^{++} on IAA loss from solution and IAAspA accumulation is very similar to that observed by Tanada (11) on Rb^+ uptake by mung bean root tips; this is of interest in that it is generally believed that IAA is not taken up as an ion but as the undissociated molecule.

These results suggest to us that the submerged roots in the absence of Ca^{++} may lose some endogenous factor essential for the uptake of IAA; that this loss is greater from excised root tips than from intact tips and can be prevented by the presence of Ca^{++}. This factor could be Ca^{++} itself as a fall in the endogenous Ca^{++} level in roots incubated in Ca^{++} free solutions has previously been observed by Jacobson and Ordin (7), who also found that this fall is prevented by the presence of Ca^{++} in the medium.

Why Ca^{++} has no effect on the accumulation of IAAspA by roots suspended on the surface of the solution or roots breaking the surface is not clear. It is possible that under such conditions the postulated factor essential for uptake does not fall below the optimal level. The relatively low accumulation of IAAspA under these conditions may be due to a more restricted access of IAA to suspended tissues than to submerged tissues.

Summary

The results have shown that while the tips of intact roots incubated in IAA solution rapidly accumulate indoleacetylaspartic acid they largely lose this ability on excision. This ability is restored if sucrose and Ca^{++} or certain other divalent cations are added to the medium. The uptake of IAA from solution, which is low in the absence of Ca^{++}, is also increased in its presence; the stimulatory effect of Ca^{++} on IAA uptake is parallel to its effect on indoleacetylaspartic acid accumulation. However, from these results it cannot be concluded whether Ca^{++} affects the uptake process directly or indirectly by affecting the rate of metabolic removal of the accumulating IAA within the tissues.

It is suggested that the Ca^{++} maintains the tissues in a healthy state essential for normal uptake, that when the tissues are placed in Ca^{+}-free media, some endogenous factor falls below a level necessary to support uptake. This loss appears to be greater from excised tips submerged than from excised tips suspended on the surface of the solution or tips of intact roots. It is further suggested that Ca^{++} and sucrose in solution prevent this loss. In any case, it seems obvious that studies on the metabolism of IAA by roots, in media lacking Ca^{++} and sucrose have been carried out under suboptimal conditions.

Literature Cited

1. Andreae, W. A. and N. E. Good 1955. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380–382.
2. Andreae, W. A. and M. W. H. van Ysselstein 1956. Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetylaspartic acid. Plant Physiol. 31: 235–240.
3. Andreae, W. A. and M. W. H. van Ysselstein 1960. Studies on 3-indoleacetic acid metabolism. VI. 3-indoleacetic acid uptake and metabolism by pea roots as compared to pea epicotyls. Plant Physiol. 35: 225–232.
4. Bonner, J. and F. T. Addicott 1937. Cultivation in vitro of excised pea roots. Bot. Gaz. 99: 144–170.
5. Burstrom, H. 1954. Studies on growth and metabolism of roots. X. Investigations of the calcium effect. Physiol. Plantarum 7: 332–342.
6. Fang, S. C., P. Theisen, and J. S. Butts 1959. Metabolism of applied indoleacetic acid-1-C^{14} in plant tissues as affected by light and 2,4-D treatment. Plant Physiol. 34: 26–32.
7. Jacobson, L. and L. Ordin 1954. Organic acid metabolism and ion absorption in roots. Plant Physiol. 29: 70–75.
8. Leggett, J. E. and E. Epstein 1956. Kinetics of sulfate absorption by barley roots. Plant Physiol. 31: 222–226.
9. Siegel, S. M. and A. W. Galston 1953. Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. Proc. Natl. Acad. Sci., U. S. 39: 1111–1118.
10. Sutcliffe, J. F. 1954. The absorption of potassium ions by plasmolysed cells. Jour. Exp. Bot. 5: 215–231.
11. Tanada, T. 1955. Effect of U.V. radiation and calcium and their interaction on salt absorption by excised mung bean roots. Plant Physiol. 30: 221–225.
12. Torrey, J. G. 1954. The role of vitamins and micronutrient elements in the nutrition of the apical meristem of pea roots. Plant Physiol. 29: 279–287.
13. Viets, F. G. 1944. Calcium and other multivalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19: 466–486.

STUDIES ON 3-INDOLEACETIC ACID METABOLISM. VI. 3-INDOLEACETIC ACID UPTAKE AND METABOLISM BY PEA ROOTS AND EPICOTYLS

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For several years we have studied the metabolism of applied indoleacetic acid (IAA) by plant tissues to determine whether growth stimulation or inhibition by IAA could be in any way related to its metabolism. So far we have been chiefly concerned with IAA metabolism by pea epicotyls, and in previous publications (*2*, 3, 4) have suggested that the toxic action of IAA might be related to the accumulation of free IAA in the tissues which appeared in turn to be related to the rate of indoleacetylaspartic acid (IAAspA) formation. Both IAAspA formation and growth became maximal at the same concentration of applied IAA (about 0.6×10^{-4} M). With lower concentrations almost all the IAA found in the tissues was present as IAAspA; only with the higher, growth inhibitory concentrations, did free IAA accumulate in the tissues to any extent.

To study more fully this apparent relationship between growth inhibition and IAA accumulation in plant tissues, we have turned our attention to pea roots. For many years it has been known that roots are over a thousand times more sensitive than epicotyls to growth inhibition by IAA. We, therefore, have attempted to determine whether this sensitivity could be related to a greater accumulation of IAA due either to an increased uptake of IAA, as compared with epicotyls, or to a less active metabolism via degradation or conjugation. Uptake in these studies was equated with loss from solution; degradation was considered to be the amount of IAA lost from solution which could not be accounted for as Salkowski reactive indole compounds in the tissues. Other workers (18) have shown that in tissue breis IAA is oxidized by the IAA-oxidase system, and it is probable that in tissues, IAA undergoes oxidative decarboxylation by the same enzyme system (9, 10).

The Salkowski reactive compounds found in the IAA treated roots are almost entirely IAA and IAAspA (*2*, 5, 9) although, as in epicotyls (12), small amounts of other derivatives are detectable. As shown below, we could find no evidence of the stable IAA-protein complex described by Siegel and Galston (16).

The present paper is concerned with the uptake, conjugation, and degradation of IAA in root tips. It also is concerned with a comparison between these findings and those of epicotyls, part of which has been published previously (5). A subsequent paper will deal with the relationship of these metabolic processes to the growth inhibitory action of applied IAA.

METHODS

Details of the methods used for raising plants and for incubating the tissues have been described in previous papers (4, 5). In some experiments, the tissues, after incubation, were transferred to a moist chamber which consisted of a large Petri dish containing a layer of moist cellocotton and a small Petri dish as receptacle for the tissues. The methods of determining IAA and IAAspA in the incubating medium and tissues have also been described in these papers.

The procedures used with roots differed in certain aspects from those used with epictoyls. These changes were necessary because roots are metabolically more active than epictoyls and more sensitive to their environment. These differences are as follows:

A. The sample weight of root tips was only 1/200 that of epicotyls but correspondingly smaller solution volumes were used except where specified so that in both cases the solutions lost about 50 % of their IAA content during 24 hours. In most experiments twelve 5 mm root tips (about 65 mg) were added to 20 ml of solution while with epicotyls 10 g of 2-inch sections were added to 500 ml of solution. The results on roots and epicotyls were then compared on a per gram fresh weight basis.

B. It was shown in a previous paper (5) that the accumulation of IAAspA and the uptake of IAA by root tips were both greatly diminished on excision and that Ca^{++} and sucrose restored these processes completely. On the other hand, Ca^{++} and sucrose were without effect on the IAA uptake and metabolism by epicotyls. Ca^{++} and sucrose were therefore added only to the solutions in which root tips, but not epicotyls, were incubated.

C. The pH of the medium used with roots was higher (pH 5.2) than that with epicotyls (pH 4.6). Figure 1 shows that with roots the rate of IAA loss from solution and IAAspA accumulation are both optimal at pH 5.2. No such pH dependence could be noted with epicotyls over the same pH range.

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[2] Contribution 161, Pesticide Research Institute, Research Branch, Canada Agriculture, University Sub Post Office, London, Ontario.

With epicotyls a pH of about 4.6 was selected as this pH minimizes bacterial contamination of the kind which markedly accelerates IAA destruction in solution.

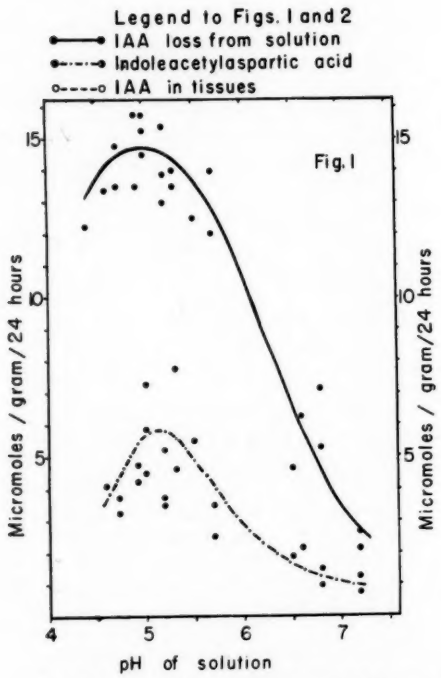
D. Although antibiotics were added to the incubation medium of both roots and epicotyls (streptomycin and penicillin did not affect the metabolism of IAA), it was necessary to autoclave the medium used for roots. The presence of sucrose and the higher pH made this medium much more susceptible to bacterial contamination which was readily detected by the appearance of turbidity in the medium. The accumulation of IAA or IAAspA in the tissues was not significantly affected in contaminated media but the results on the loss of IAA from solution became most erratic. Consistently sterile conditions prevailed throughout the experimental period if roots from sterile seedlings were used; this precaution gave essentially the same results as the non-sterile tissues manipulated by the simplified procedure.

The two tissues differed in age; epicotyls were taken from 7-day old seedlings while root tips were taken from 2-day old seedlings. These tissues were selected because they were of the same stage of development as tissues used in growth studies, and thus, IAA metabolism could be directly compared to its effect on growth. The results of growth studies will be presented in a subsequent paper.

Results

I. IAA Metabolism by Roots. A. *Metabolic changes after incubation for 24 hours.* Figure 2 shows that the loss of IAA from solution increases constantly over the entire concentration range studied, while the IAAspA accumulation increased similarly only up to 2×10^{-4} M IAA; at this concentration the rate of conjugation was maximal and declined at higher concentrations. At optimum conditions of IAAspA accumulation the concentration of IAAspA reached 1.6 % of the dry weight. Below 4×10^{-4} M of applied IAA, free IAA could not be detected in the tissues, but was present at higher concentration.

B. *Metabolic changes at intervals over 24 hours.* Quite a different pattern of IAA metabolism is obtained during the early hours of incubation. For the short-term experiments only one-quarter the amount of solution was used; twelve tips were incubated in 5 ml solution in 50 ml Erlenmeyer flasks. This change of the procedure still provided about the same solution depth and enough IAA so that after 14 hours



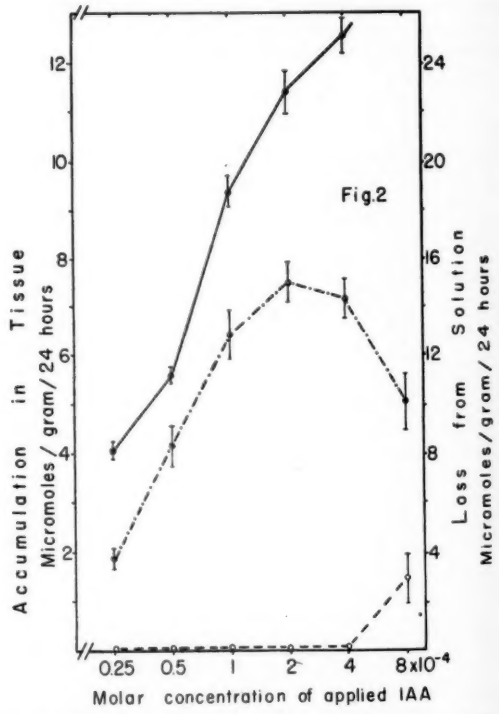


Fig.2
Accumulation in Tissue Micromoles / gram/ 24 hours
Loss from Solution Micromoles/gram/ 24 hours
Molar concentration of applied IAA

Fig. 1. The effect of pH on the IAA loss from solution and the accumulation of indoleacetylaspartic acid by root tips incubated in 10^{-4} M IAA solution for 24 hours.

Fig. 2. The IAA lost from solution and the accumulation of indoleacetylaspartic and IAA by root tips incubated in IAA solutions of various concentrations for 24 hours. The vertical lines represent twice the standard error of the mean of three experiments.

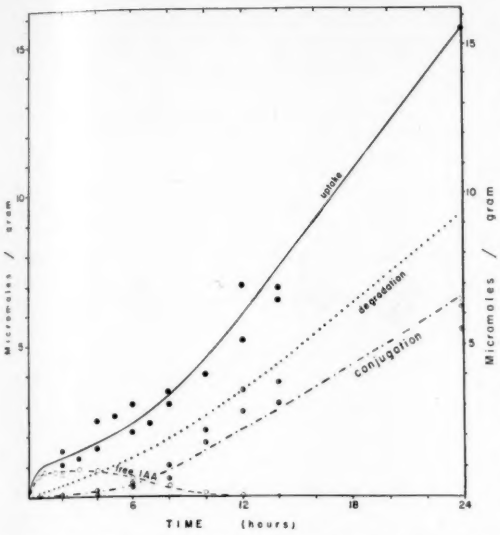


FIG. 3. The loss of IAA from solution and accumulation of IAA and indoleacetylaspartic acid in pea root tips during 24 hours' incubation in 10^{-4} M IAA. The degradation line represents the difference between the IAA lost from solution and the IAA and indoleacetylaspartic acid accumulated in the tissues.

of incubation only 50 % of the initial IAA was left in solution, but the accuracy of determining the IAA lost from solution was increased four-fold.

Figure 3 shows that root tips during the first half hour of incubation in 10^{-4} M IAA take up IAA very rapidly. The IAA lost from solution can be recovered almost quantitatively from the tissues as accumulated free IAA. At 1 hour the accumulated free IAA represents about a ten-fold greater concentration within the tissues than in the surrounding medium. The tissue IAA remains at this high level for 2 to 3 hours, after which it diminishes; by the 12th hour it has completely disappeared from the tissues. A similar proportionate high initial IAA accumulation within the tissues was observed at all concentrations of applied IAA (table I). In the presence of 10^{-3} M KCN the IAA content of the tissues rose only to the concentration of the external solution.

After the initial half hour of incubation, the rate of IAA uptake is greatly diminished for about five hours. During this time the amount of IAA lost from solution can no longer be accounted for completely as Salkowski reactive compounds in the tissues. This indicates that the degradative activity has commenced. However, degradation does not reach maximum activity until about the sixth hour of incubation. There was no evidence of IAAspA formation until after the second hour. This was confirmed by chromatography (fig 4). Because of the relatively long lag period required for degradative and conjugative activity to commence, it is possible to separate the initial uptake of IAA from the subsequent continuous uptake. After the initial uptake is complete the rate of subsequent uptake of IAA is dependent upon the rate of its metabolic removal from the tissues.

The initially accumulated IAA which subsequently disappears from the tissues was metabolized by conjugation and degradation as soon as these processes became active. This can be seen in an experiment in which the root tips, after 2 hours incubation in IAA solution, were transferred to a moist chamber (fig 5). Within the next 4 hours about 70 % of the IAA in the tips disappeared and could be accounted for completely as IAAspA; the residual IAA in the tips was not conjugated but degraded to Salkowski unreactive compounds during the next 24 hours. It should be noted that while no degradative loss of the accumulated IAA was observed during the first 4 hours in the moist chamber, some degradative activity was apparent before the sixth hour in the previous experiment where the tips were incubated in solution (fig 3). This suggests that the relatively small amount of degradation observed during the early hours in solution may be limited to the cut surface of the tissues and that the process requires a longer period to become active within the tissues. This raises the question as to whether loss from solution can be equated with uptake or whether some extracellular oxidation may occur. At the moment there is some doubt if all the IAA degraded is first taken up by the tissues. However, there can be little secretion of enzymes into the solution as IAA loss from solution ceases when the tissues are removed; this was also found to be the case with epicotyls (4).

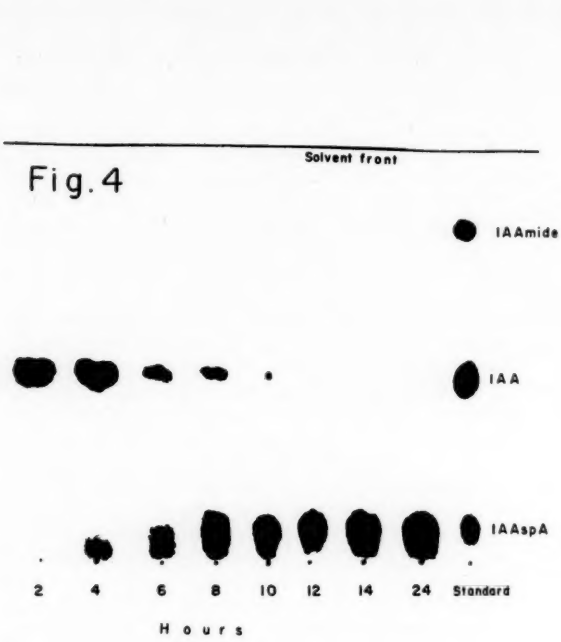
There was no evidence of the IAA-protein formation as described by Siegel and Galston (16). This complex was reported to form rapidly within the first 30 minutes of incubation in pea root tips treated with IAA, and to accumulate to about the same concentration as free IAA. Siegel and Galston obtained their complex by precipitating the proteins of the breis with trichloroacetic acid. The precipitated IAA-protein complex was then dissolved in 0.1 *N* NaOH; this alkaline extract gave a positive Salkowski reaction. Under the conditions of our studies any IAA

TABLE I

IAA CONTENT OF ROOT TIPS AND EPICOTYLS [1]

CONCENTRATION OF APPLIED IAA μM/ML	CONCENTRATION OF IAA IN TISSUES μM/G	
	ROOT TIPS	EPICOTYLS
0.05	0.48	0.031
0.10	0.87	0.074
0.20	2.00	0.180
0.30	3.00	0.250
0.40	3.90	0.415

[1] After 2 hours incubation in various concentrations of applied IAA.



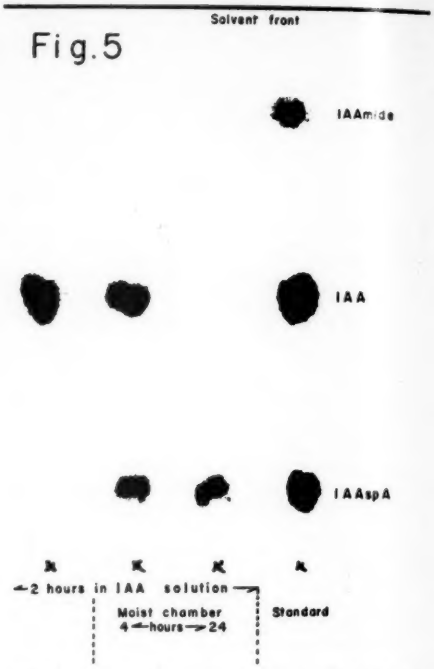


FIG. 4. Chromatogram of root tip extracts after 2, 4, 6, 8, 10, 12, 14, and 24 hours incubation in 10^{-4} M IAA solution. The standard, at the right, contains indoleacetamide (IAAmide), IAA, and indoleacetylaspartic acid (IAAspA). The chromatogram was sprayed with Ehrlich reagent.

FIG. 5. Chromatogram of extracts of root tips incubated in 10^{-4} M IAA solution for 2 hours; from the left first extracted immediately, next in a moist chamber for 4, and last for 24 hours before extraction. The standard, at the right, contains indoleacetamide (IAAmide), IAA, indoleacetylaspartic acid (IAAspA). The chromatogram was sprayed with Ehrlich reagent.

present in the tissues was completely ethanol extractable; an alkaline extract of the proteinaceous residue, after ethanol extraction, gave a negative Salkowski reaction. This was true whether looked for in excised root tips incubated for 3 to 24 hours; whether Ca^{++} and sucrose were added to the medium, or whether intact roots or root tips were used. In our opinion, the IAA-protein complex is an artifact of the trichloroacetic acid precipitation procedure. The experimental evidence for the metabolic nature of this process is not convincing as uptake of IAA from solution by roots is also a metabolic process which would be inhibited by the same metabolic inhibitors.

II. IAA METABOLISM BY PEA EPICOTYLS. Our previous publication on the IAA metabolism of pea epicotyls (4) was primarily concerned with metabolic changes after 24 hours incubation. At low concentrations of applied IAA (below 10^{-4} M IAA) little free IAA was found in the tissues after 24 hours. It was not suspected at that time that free IAA might appear and again disappear during the initial hours of incubation. The time course was studied but only at 2, 4, 8, and 16 hours and with a single concentration of 1.72×10^{-4} M IAA. This was supraoptimal for IAAspA formation, so that IAA persisted in the tissues. Our recent observations with root tips have compelled us to reinvestigate the time course for epicotyls. We, therefore, have extended the experiments over a wider concentration range and closer time intervals to allow comparison with the results on roots (table I and fig 6).

For the short term experiments, only 2 g of epicotyls were incubated in 40 ml solution in 125 ml Erlenmeyer flasks. This change of procedure increased the accuracy of determining the IAA loss from solution without significantly affecting the experimental conditions of incubation.

Figure 6 shows that the loss of IAA from solution was about linear with time for the highest concentrations of applied IAA. For the lowest concentrations (0.25×10^{-6} M), however, there was a slow loss from solution over a period of several hours. This loss then almost ceased until the 6th hour when the loss again increased. During the first 2 hours with the low concentration, almost all the IAA lost from solution could be accounted for as free IAA in the tissues. With all concentrations the free IAA

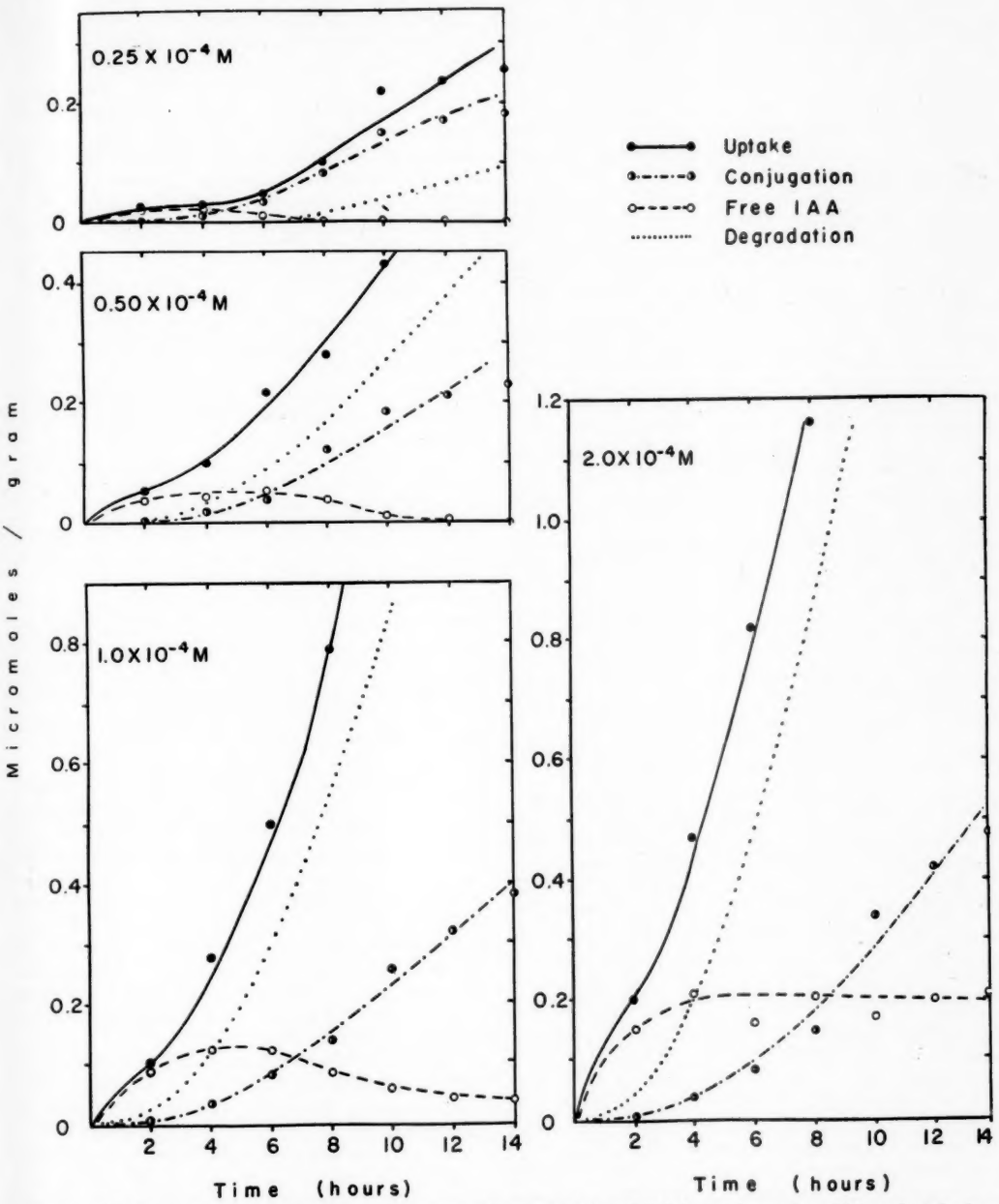


FIG. 6. The loss of IAA from solution and accumulation of IAA and indoleacetylaspartic acid in epicotyls during 14 hours of incubation in 0.25, 0.5; 1.0 and 2.0 $\times$ 10^{-4} M IAA solution. The degradation line represents the difference between the IAA lost from solution and the IAA and indoleacetylaspartic acid accumulated in the tissues.

content of the tissues rose over a period of 4 to 6 hours, until it reached a concentration equal to or less than that in the external solution (table I). This equilibrium was also established in the presence of 10^{-3} M KCN. On prolonged incubation the IAA content of the tissue decreased and disappeared by about the twelfth hour except with 2×10^{-4} M IAA, a concentration supraoptimal for IAAspA formation. This fall is associated with the conjugative activity. The lag period of conjugation, unlike that of degradation, is independent of the concentration of IAA applied; in every case conjugative activity commenced about the second hour and became maximal at about the sixth hour.

Degradative activity, on the other hand, showed a lag period which decreased with increasing concentration of applied IAA. While some degradative loss is apparent by the 2nd hour with 2×10^{-4} M IAA, none could be observed even at the 6th hour with 0.25×10^{-4} M IAA.

The results indicate clearly that after the IAA content of the tissues has attained equilibrium, the rate of the subsequent uptake, as in roots, is dependent upon the rate of the metabolic removal via conjugation and degradation. As the initial uptake of IAA, on the other hand, was found to be insensitive to cyanide, and as IAA does not accumulate in the tissues beyond the concentration in solution, it would seem that the uptake process per se is a non-metabolic process. In an earlier paper (4) it was concluded that the uptake of IAA by pea epicotyls was entirely metabolic for no IAA or IAAspA could be detected in the tissues incubated under pure nitrogen. In these experiments, the tissues and IAA solutions were placed in flasks from which the air was evacuated and replaced by pure nitrogen. At present there is no adequate explanation why, under these conditions, IAA did not enter the tissues by a physical process. In a recent modified experiment in which tissues were maintained under anaerobic conditions by placing them in flasks completely filled with boiled and cooled solution and stoppered under expulsion of any air space, the IAA content of the tissues rose to the same level as under aerobic conditions.

Discussion

In these experiments we have attempted to set up metabolic balance studies for uptake and metabolism of IAA by pea roots and epicotyls during 24 hours incubation. The results indicate that the differences between IAA metabolism by roots and epicotyls are chiefly of quantitative nature. Both degradation and conjugation, once the mechanisms have been adapted, are more rapid with roots than epicotyls.

The uptake mechanism of IAA, however, appears to be distinctly different between roots and epicotyls. Considering the function of these two tissues, this is not surprising. In roots there is a rapid metabolic uptake leading to accumulation of IAA in the tissues in excess over the external concentration, while in epicotyls there apparently is a physical process by which IAA concentration of the tissues does not exceed that in solution (table I). The kinetics of the rapid metabolic uptake by root tips appear to be analogous to the uptake of galactosides by induced *Escherichia coli* bacteria as studied by Cohn and Monod (7). These workers ascribed galactoside uptake to the catalytic action of a galactoside permease system. The observed high galactoside accumulation inside the tissues results from the greater rate of the entry reaction over the exit reaction; thus the accumulated galactosides may be considered as steady state intermediates. This concept has led Miller (14) to derive the expression:

$$(S_i) = \frac{K_e}{K_e} (S_o)$$

where (S_i) and (S_o) are the internal and external concentrations at thermodynamic equilibrium, and K_e and K_e are constants characteristic of the permease system. The uptake of IAA by roots as given in table I can be seen to follow this expression if K_e/K_e is taken as approximately ten.

This accumulation could only be observed during the early periods of incubation before the metabolic processes for IAA removal have been activated. Once conjugation and degradation have become adapted the IAA disappears from the tissues unless supraoptimal concentrations for IAAspA formation are applied. When an equilibrium between the internal and external IAA concentration has been attained (either actively as in roots or passively as in epicotyls) the rate of uptake slows down until the metabolic processes of IAA destruction and conjugation become adapted. The rate of continued IAA uptake is then limited by the rate of its metabolic removal from within the tissues.

The uptake of IAA by other plant tissues has been previously discussed by Albaum et al (1) and Sutter (17), who studied Nitella cells and cucumber hypocotyls, respectively, and considered IAA uptake a purely physical process. Reinhold (15) concluded from her studies with carrot root discs that two processes are involved, an initial physical uptake followed by a continued slow metabolic uptake which she attributed either to an active transport of IAA into the cells or else to its metabolic removal. Our results, as just shown, provide evidence for the latter alternative.

Both degradation and conjugation appear to be auxin inactivating processes which may occur in different areas of the tissue. This is suggested by results on the metabolism of accumulated IAA (fig 5). There is also some histological evidence in the literature that the areas of degradation and conjugation are located in separate regions. First, Jensen (13) has shown that the peroxidase activity in root sections of broad beans is limited to the root cap, epidermis, and vascular elements. Since the IAA-oxidase system is composed of a hydrogen peroxide producing system and a peroxidase (10), and since degradation of IAA by intact tissues probably takes place via the IAA-

oxidase system, it is likely that the degradative activity is limited to these areas of peroxidase activity. Second, Ebert (8) has shown that roots incubated in 10^{-3} M IAA for 10 hours accumulate a Salkowski reactive compound in the central areas; little or none could be detected in the epidermis, root hair or root cap. Ebert considered this Salkowski reactive compound to be IAA bound to tissues as it persisted in the tissues even if rinsed in running water for several days. From our studies it seems certain, however, that Ebert's Salkowski reactive compound was chiefly IAAspA. We have previously reported (2) that IAAspA is resistant to leaching and metabolic degradation.

In view of these histological studies it is suggested that while degradation may be limited to the root cap, epidermis, and vascular elements, conjugation occurs primarily in the cortical areas. It could also be argued that the conjugating and degrading sites are located in different areas of the same cell, but in our opinion, histological evidence favours the former concept. However, the possibility must not be excluded that besides IAA-oxidase, other, as yet unknown, systems of degradation might exist.

If these two reactions take place in different areas of the tissue, then the accumulation of IAA may occur in one or the other areas separately. Since degradation did not attain a maximal rate over the concentration range studied, it is unlikely that IAA could be found in the degradative areas after this process has become adapted. On the other hand, as conjugative activity reaches a maximal rate with high concentrations of applied IAA (10^{-4} M for epicotyls and 2×10^{-4} M for root tips) it stands to reason that with higher concentrations, IAA will accumulate in the conjugating areas. It has in fact been shown that IAA which has accumulated in epicotyls incubated in 1.72×10^{-4} M IAA for 16 hours is subsequently completely conjugated when the tissues are placed in a moist chamber (4); there was no evidence of degradation.

Degradation in the present studies has only been determined indirectly as the IAA lost from solution which could not be accounted for as Salkowski reactive compounds in the tissues. Fang et al (9), by means of carboxyl labeled IAA, have shown that degradation involves oxidative decarboxylation and over a period of 19 hours accounts for almost all the IAA metabolized which could not be recovered from the tissues as IAA or IAAspA. Quantitatively they confirmed our results that in epicotyls over 80 % of the IAA lost from solution is degraded while about 15 % is retained in the tissues as free IAA or IAAspA after 24 hours incubation (4). With roots they found degradation accounted for about 93 % and conjugation for only 7 % of the IAA lost from solution. These latter results also agree with our experiments (5) in which Ca^{++} was absent from the medium. In the present study where Ca^{++} had been added, degradation accounted for only 74 % and conjugation for 26 % of the loss after 24 hours. From the present experiments, however, it is clear that the proportions between degradation and conjugation are not absolute and depend on the time of incubation and the concentration of IAA applied; during the first 6 hours in epicotyls treated with 0.25×10^{-4} M IAA all the IAA metabolized is conjugated; there is no evidence of degradation until after the 6th hour.

There has been considerable discussion as to the significance of degradative activity in the tissues. This reaction has been largely studied in tissue breis, where it is active, unlike the formation of IAAspA which has not yet been demonstrated in vitro. Galston (10) postulated that IAA-oxidase not only metabolizes applied IAA, but also controls the endogenous auxin level of untreated tissues thus explaining aging phenomena, auxin rhythm, and lateral bud inhibition. Briggs et al (6), on the other hand, consider that IAA-oxidase activity is purely a cut surface phenomenon, as the amount of applied IAA recovered after transport through tissue segments was unaffected by the length of tissue through which the IAA had passed. Neither Reinhold (15) nor we (4), however, were able to find any proportionality between the area of cut surface and oxidase activity. We believe that rather than being limited to the cut surface, this process, once adapted, occurs in the areas which have been shown to possess peroxidase activity, but that because of the long lag periods found with low concentrations of applied IAA this process may play no part normally in the tissues studied.

It should be noted, that equating loss from solution with degradation, Galston and Dalberg (11) have reported a 10 to 20 minute lag period for this process to become adapted in epicotyls incubated in 2×10^{-4} M IAA. As seen in the present studies, loss from solution cannot be equated with destruction, for even after 2 hours incubation in this concentration of IAA, 80 % of the IAA lost from solution could be found as IAA in the tissues.

These studies on IAA uptake and metabolism were carried out concurrently with studies on the inhibitory action of IAA on growth. A subsequent paper will deal with the relationships observed. It might be said at this point, however, that while the IAA content of roots has been shown (fig 2) to be considerably greater than that of epicotyls (fig 6) during the first half hour incubation in IAA, it is not believed that this difference can account for the greater inhibitory action of IAA on root growth.

Summary

I. Root tips and epicotyl segments of pea seedlings were incubated in IAA solutions for various lengths of time. Quantitative measurements were made to determine the IAA lost from solution and the free IAA and indoleacetylaspartic acid in the tissues. Besides IAA, only indoleacetylaspartic acid occurred in the tissues to any extent as Salkowski reactive compounds; there was no evidence of any IAA-protein complex. The formation of indoleacetylaspartic acid was referred to as the conjugation

process. The loss of IAA by degradation was followed indirectly as the difference between the IAA lost from solution and the Salkowski reactive compounds of the tissues.

II. During the early period of incubation the IAA loss from solution could be almost quantitatively recovered in the tissues as free IAA, as both conjugative and degradative activity require some time to become apparent. For conjugation a period of about two hours is required for any evidence of activity, while maximal activity is not attained for 4 to 6 hours. These time relationships are independent of the applied IAA concentration. For degradation, on the other hand, the period of adaptation is inversely proportional to the concentration of applied IAA. The disappearance of IAA from the tissues appears to be related almost entirely to the conjugative activity. It is suggested that the degradative activity is limited to a small area of tissue, probably the epidermis and root cap. Both the degradative and conjugative activity are more active processes in roots than in epicotyls.

III. The uptake of IAA by roots appears to be an active, cyanide sensitive process whereby roots accumulate IAA to ten times the external concentration. The uptake of IAA by epicotyls, on the other hand, appears to be a physical process in which the IAA content of the tissues does not exceed that in solution. In both cases, the IAA content of the tissues was proportionate to the concentration of IAA applied. The rate of continuous uptake in both roots and epicotyls was limited by the rate of metabolic removal of IAA within the tissues.

IV. Because of the rapid active accumulation of IAA by root tips, the IAA content of the tips after half an hour of incubation was much greater than in epicotyls. This difference gradually decreased. After 10 hours little or no IAA was detectable in either tissue unless a concentration of IAA supra-optimal for indoleacetylaspartic acid formation was applied.

Literature Cited

1. Albaum, H. G., S. Kaiser, and H. A. Nestler 1937. The relation of hydrogen-ion concentration to the penetration of indoleacetic acid into Nitella cells. Amer. Jour. Bot. 24: 513–518.
2. Andreae, W. A. and N. E. Good 1955. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380–382.
3. Andreae, W. A. and N. E. Good 1957. Studies on 3-indoleacetic acid metabolism. IV. Conjugation with aspartic acid and ammonia as processes in the metabolism of carboxylic acids. Plant Physiol. 32: 566–572.
4. Andreae, W. A. and van M. W. H. Ysselstein 1956. Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetylaspartic acid. Plant Physiol. 31: 235–240.
5. Andreae, W. A. and van M. W. H. Ysselstein 1960. Studies on 3-indoleacetic acid metabolism. V. The effect of calcium ions on the 3-indoleacetic acid uptake and metabolism by pea roots. Plant Physiol. 35: 220–224.
6. Briggs, W. R., T. A. Steeves, I. M. Sussex, and R. H. Wetmore 1955. A comparison of auxin destruction by tissue extracts and intact tissues of the fern *Osmunda cinnamomea*. Plant Physiol. 30: 148–155.
7. Cohen, G. N. and J. Monod 1957. Bacterial permease. Bact. Review 21: 169–194.
8. Ebert, V. A. 1955. Versuche über die quantitative Bestimmung der Indolessigsäure und ihre Lokalisierung in Geweben der Pflanzen. Phytopath. Zeit. 24: 216–242.
9. Fang, S. C., P. Theisen, and J. S. Butts 1959. Metabolic studies of applied indoleacetic acid-1-C^{14} in plant tissues as affected by light and 2,4-D treatment. Plant Physiol. 34: 26–32.
10. Galston, A. W. 1956. Some metabolic consequences of the administration of indoleacetic acid to plant cells. Proc. Symp. Wye College, 219–233.
11. Galston, A. W. and L. Dalberg 1956. The adaptative formation and physiological significance of indoleacetic acid-oxidase. Amer. Jour. Bot. 41: 373–380.
12. Good, N. E. and W. A. Andreae 1956. Studies on 3-indoleacetic acid metabolism. II. Some products of the metabolism of exogenous 3-indoleacetic acid in plant tissues. Plant Physiol. 31: 231–235.
13. Jensen, W. A. 1955. The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. Plant Physiol. 30: 426–432.
14. Miller, D. M. 1960. The osmotic pump theory of selective transport. Biochim. Biophys. Acta 37: 448–462.
15. Reinhold, L. 1954. The uptake of indoleacetic acid by pea epicotyl segments and carrot disks. New Phytologist 53: 217–239.
16. Siegel, S. M. and A. W. Galston 1953. Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. Proc. Nat. Acad. Sci. 39: 1111–1118.
17. Sutter, E. 1944. Die chemische Bestimmung des Heteroauxins und Versuche über seine Aufnahme durch die Pflanze. Ber. schweiz. bot. Ges. 54: 197–244.
18. Tang, Y. W. and J. Bonner 1947. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25.

A SECONDARY PHOTOSYNTHETIC CARBOXYLATION[1]

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INTRODUCTION

Several investigators (12, 13, 14) have confirmed Aufdemgarten's (1, 2) finding that a transient uptake of CO_2 often occurs when a leaf is illuminated in an atmosphere containing a relatively high CO_2 concentration. This uptake occurs at the onset of illumination and stands out as a prominent feature of the gas exchange during the photosynthetic induction period. Van der Veen, who used the same gas analysis methods as Aufdemgarten to study the uptake of CO_2 during the induction period, labeled this uptake of CO_2 the "initial uptake". A valuable contribution from his extensive study was the determination of conditions which would isolate this phenomenon from normal photosynthesis. Veljby (14) has essentially repeated some of Van der Veen's measurements. Some important data have come from the work of Massini (9) on the assimilation of $C^{14}O_2$ during the initial uptake. The purpose of the present paper is a restudy of this phenomenon. In this investigation O_2 as well as CO_2 were measured so that the assimilatory quotient could be determined. In order to account for the abnormally high quantum yields found by some workers, Franck (6) postulated that at the beginning of a period of illumination, when the CO_2 acceptor was absent, respiratory intermediates were photochemically reduced. The similar hypothesis which will be developed here is that the initial uptake of CO_2, which is different from the normal photosynthetic fixation of CO_2 is due to a light-induced reductive carboxylation of a respiratory intermediate.

MATERIALS AND METHODS

The heat-conductivity method of gas analysis was used to measure the photosynthetic gas exchange of Hydrangea leaves. The apparatus was similar to that described by Aufdemgarten (1, 2) for measuring time course of the rate of CO_2 assimilation in photosynthesis. A schematic diagram of the apparatus and gas flow system is shown in figure 1. In an atmosphere of air or nitrogen only the change of CO_2 content due to respiration or photosynthesis is manifest because the heat conductivity of O_2 is practically the same as that of N_2 with the result that the sensitivity to a change of O_2 content is very small. Van der Veen showed that both O_2 and CO_2 could be measured in a H_2 atmosphere. If a CO_2 trap is placed in the gas flow line between the leaf and the gas analysis chamber, only O_2 changes are measured. If this CO_2 trap is bypassed, the effect of the concurrent O_2 and CO_2 changes is measured. By performing two consecutive experiments under identical conditions, one with and one without the CO_2 trap, curves for the rate of change of O_2 content and for the rate of change of $O_2 + CO_2$ content are obtained. The curve for the rate of change of CO_2 content can be constructed by subtracting the O_2 curve from the $O_2 + CO_2$ curve. In the work to be reported here O_2 and CO_2 changes were measured in an experimental atmosphere of 4 % CO_2 and 20 % O_2 in He. The apparatus was 1.3 times as sensitive to CO_2 changes as to O_2 changes. There is a linear calibration between the voltage output of the Wheatstone bridge which was recorded with a Leeds and Northrup strip-chart recorder and O_2 and CO_2 content of the experimental atmosphere. The gas is saturated with water vapor before passing over the leaf and dried

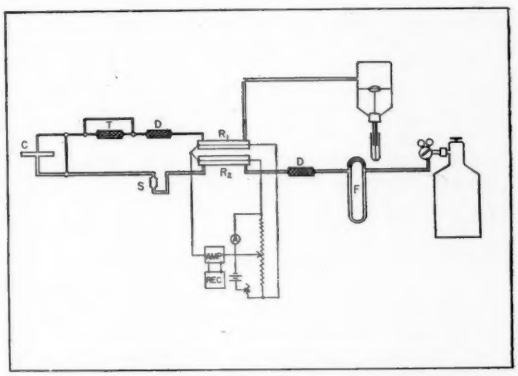

FIG. 1. Schematic diagram of apparatus. The platinum wires R_1 and R_2 form two arms of an equal arm Wheatstone bridge. These wires are heated by the current flowing through the bridge. The temperature of the wires and therefore their resistance depends on the heat conductivity of the gas mixture flowing over the wires. The composition and therefore the heat conductivity of the gas flowing over R_2 is constant while that flowing over R_1 is altered by the metabolism of the leaf. The voltage across the detecting terminals of the bridge is a measure of the composition of the gas flowing through R_1.

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by passing through $CaCl_2$ before entering the gas-analysis chamber. The gas-flow system terminated in a large jar designed to isolate the system from rapid changes of atmospheric pressure.

At the flow rates used (50 ml/min) there was a delay of about 30 seconds in the recording of a change of gas composition due to the volume between the leaf chamber and analyzing chamber. This delay limits the response of the instrument.

In order to determine the compensation point for O_2 or CO_2 on these curves (i.e., the point at which the O_2 or CO_2 exchange of respiration is exactly compensated by that of photosynthesis so that the net exchange of the gas in question is zero), the gas flow is shunted around the leaf chamber. The reading obtained represents the condition of zero gas exchange.

In most of the work the underside of a Hydrangea leaf cut from a mature greenhouse plant was illuminated. All of the stomata of a Hydrangea leaf are on the underside and are open at all times. This was determined by placing a drop of alcohol on the leaf surface and observing it penetrate the leaf through the open stomata. The alcohol will infiltrate the leaf rapidly only if its stomata are open. Ailanthus leaves were used to repeat Van der Veen's experiments on heat treatment. Hydrangea and most other plants do not show the heat treatment phenomena.

Results

A typical curve for the rate of gas exchange vs. time for a Hydrangea leaf in an atmosphere of 4 % CO_2 and 20 % O_2 in He is shown in figure 2. The rate of change of O_2 evolution rises rapidly to the compensation point when the light is turned on; it remains there momentarily before proceeding on to the steady-state rate of O_2 evolution. The rate of CO_2 assimilation initially goes through a maximum exceeding its conpensation level. During the first moment of illumination the assimilatory quotient $\left(\text{A.Q.} = \frac{\text{rate of } O_2 \text{ exchange in light minus rate in dark}}{\text{rate of } CO_2 \text{ exchange in light minus rate in dark}}\right)$ has a minimum value of about a third. During the monotonous rise to steady-state photosynthesis and at steady-state photosynthesis the quotient is unity. This investigation was concerned primarily with this anomalous assimilatory quotient which occurs during the initial minute of illumination.

Van der Veen showed that, while the steady-state rate of photosynthesis was depressed to a very low value by cooling a leaf to 0° C in 4 % CO_2, the initial uptake of CO_2 remained. The photosynthetic gas exchange for a Hydrangea leaf under these conditions is shown in figure 3.

At the low temperature of 0° C the magnitude of the initial gas exchange is markedly influenced by the duration of the previous light and dark periods. These effects on the initial O_2 evolution are shown in figure 4. The dependence of the size of the O_2 burst on the duration of the previous dark period shows a rather broad maximum at 10 minutes with practically as large an effect at 8 or 15 minutes. In curve A the dark period of 10 minutes produces the maximal O_2 burst. The initial O_2 burst is smaller in curves B and C where the dark periods were 1 minute and 40 minutes, respectively. In curve D which follows the 10-minute illumination period of curve C by a 10-minute dark period the maximal O_2 burst is restored. In each of the above curves the period of illumination preceding the dark period was of sufficient duration to produce the maximal O_2 burst in the next illumination. When the first period of illumination was less than 10 minutes, the O_2 burst at the beginning of the subsequent illumination was decreased. In each case the rate of CO_2 uptake was about three times the rate of O_2 evolution during the initial gas exchange.

Van der Veen also showed that the steady-state photosynthetic gas exchange of certain leaves could be destroyed by a careful heat treatment while an initial uptake of CO_2 remained. The CO_2 taken up at the onset of illumination was released when illumination ceased. Figure 5 shows this phenomenon with an Ailanthus leaf which had been heated to 51° C for 3 minutes. The magnitude of the initial uptake of CO_2 by the heat-treated leaf is considerably less than by the normal leaf. There are no concomitant transient O_2 changes. Although photosynthesis has been destroyed by the heat treatment, respiration proceeds at a normal rate. In cases where respiration has also been destroyed by the heat treatment no initial uptake of CO_2 occurs.

The constructed curves for the rate of change of CO_2 content in an atmosphere of 4 % CO_2 and 20 % O_2 in He have been duplicated by the direct measurement of CO_2 changes in an atmosphere of 4 % CO_2 in air. The substitution of He for N_2 does not affect the metabolism of the leaf.

Whereas the initial uptake of CO_2 was a common feature in the induction period of leaves in atmospheres of high CO_2 concentration, it was not observed with algae. Scenedesmus showed a smooth monotonous transition from the dark to the light gas exchange and chlorella exhibited the initial burst of CO_2 first described by Emerson and Lewis (5). In low CO_2 concentration a very brief shoulder at the CO_2 compensation level rather than a net uptake of CO_2 has been observed at the onset of illumination. This shoulder is probably the result of a momentary cessation of respiratory CO_2 evolution before the photosynthetic assimilation has begun. Gaffron (8) has studied this phenomenon in detail, showing with chlorella, where the initial uptake of CO_2 does not occur, that a two-second illumination will compensate respiration for 40 seconds. The curves of McAllister and Myers (10) with young wheat plants in low CO_2 indicate the same phenomenon. Even though this shoulder at the compensation level resembles a small, rapid initial uptake of CO_2 it is probably not the result of a true carboxylation and will not be considered here.

Discussion

The assimilation of CO_2 during the first minute of illumination of a leaf in 4 % CO_2 is different from the normal photosynthetic carboxylation of ribulose diphosphate. The assimilatory quotient (A.Q.) of the transient process is about one-third whereas that of photosynthesis is close to unity. Four percent CO_2 at 0° C inhibits the steady-state rate of photosynthesis to a very low value without concomitantly inhibiting the initial carboxylation. Massini (9) has shown that iodacetamide inhibits photosynthesis to a much greater degree than the initial uptake of CO_2 and that the dependence of the initial uptake on light intensity saturates at a much lower light intensity than does photosynthesis. A large initial uptake of CO_2 occurs only when the CO_2 content is considerably in excess of that required for photosynthesis. Since the carboxylation which occurs during the first minute of illumination shows so many differences from normal photosynthesis it is considered to be a secondary photosynthetic carboxylation.

The experimental contribution of this paper is the measurement of the A.Q. during this transitory, light-induced carboxylation and the manner in which the initial gas exchange at 0° C in 4 % CO_2 depends upon the previous dark and light periods. Of the possible mechanisms of this carboxylation the author regards the most probable one to be the reductive

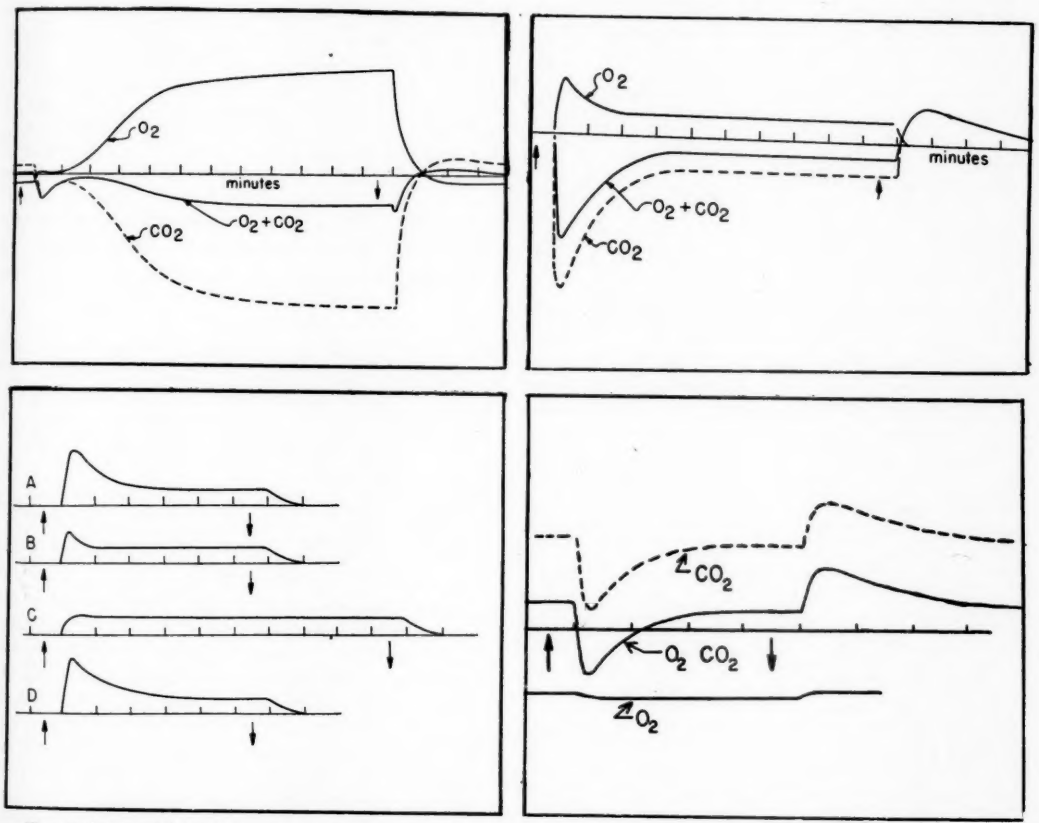


Fig. 2 (*upper left*). Rate of gas exchange vs. time for a Hydrangea leaf at room temperature in an atmosphere of 4 % CO_2 and 20 % O_2 in He. Gas evolution is plotted above and consumption below the abscissa scale. Arrows mark the beginning and end of illumination. The steady-state rate of O_2 evolution and CO_2 uptake is 18 μl/min in a flow rate of 50 ml/min.

Fig. 3 (*upper right*). Rate of gas exchange vs. time(min) for a Hydrangea leaf at 0° C in an atmosphere of 4 % CO_2 and 20 % O_2 in He.

Fig. 4 (*lower left*). Rate of O_2 evolution vs. time (min) for a Hydrangea leaf at 0° C in an atmosphere of 4 % CO_2 and 20 % O_2 in He. Curve A: dark 10 minutes. Curve B: dark 1 minute. Curve C: dark 40 minutes. Curve D: dark 10 minutes.

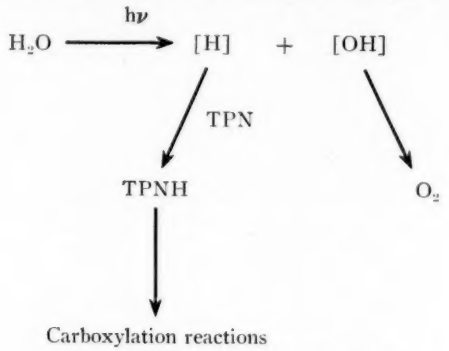
Fig. 5 (*lower right*). Rate of gas exchange vs. time (min) for heat-treated Ailanthus leaf at room temperature in an atmosphere of 4 % CO_2 and 20 % O_2 in He. The leaf had been heated to 51° C for 3 minutes.

carboxylation of pyruvate via reduced triphosphopyridine nucleotide (TPNH). This explanation which is based on biochemical pathways known to occur in higher plants accounts for the experimental data presented here as well as the work of previous investigators.

The malic enzyme which catalizes the reductive carboxylation of pyruvate to malate has been demonstrated in a number of higher plants (4).

$$\text{pyruvate}^- + CO_2 + \text{TPNH} \quad \text{malate}^{--} + \text{TPN}^+$$

The reaction whose equilibrium constant favors decarboxylation is driven to the right by the light-induced reduction of TPN, the high concentration of



CO_2 and the subsequent removal of the malic acid. Under the normal steady-state conditions of photosynthesis the photochemically produced TPNH is utilized for the reductive processes of photosynthesis. At the beginning of the induction period, however, when the normal photosynthetic intermediates are absent the photochemically produced TPNH may reduce respiratory intermediates such as pyruvic acid. The TPNH which does not react in carboxylation reactions reacts back with [OH] so that the uptake of CO_2 is coupled to the evolution of O_2 from [OH]. The A.Q. for the proposed reductive carboxylation is one-half.

In the present experiments it was shown that for a maximal O_2 burst at 0° C in 4 % CO_2 there must have been a previous illumination of at least ten minutes followed by a dark period of ten minutes. The concentration of pyruvate, the substrate for the reductive carboxylation, present at the onset of illumination will determine the size of the O_2 burst. The most plausible explanation is that during the 10-minute light period carbohydrate is accumulated to a steady-state light concentration by the low rate of photosynthesis occurring under the conditions of the experiment (3 % of the rate at 25° C). During the dark, pyruvate is formed by respiratory reactions from the carbohydrate accumulated during the previous light period. The pyruvate concentration increases to a maximum concentration during the first 10 minutes of the dark period. For longer dark periods the depletion of the pool of previously accumulated carbohydrate results in a decreasing concentration of pyruvate as the rate of respiratory utilization of pyruvate becomes more rapid than the rate of formation.

The suggestion that the transitory light-induced carboxylation yields malate receives support from the work of Massini (9) with Dahlia leaves which exhibit the initial uptake of CO_2. He found that after a 10-minute dark period the concentration of malic acid rose to its steady-state light value in the first 15 seconds of illumination. After the 10 minute dark period which preceded the illumination, the induction period of the leaf would have been long enough to preclude much CO_2 fixation by normal photosynthesis. During this time, however, $C^{14}O_2$ was being assimilated by what we have called the secondary photosynthetic carboxylation and C^{14} labeled malic appeared. Massini (9) concluded the initial uptake of CO_2 was not due to a carboxylation leading to malate on the basis of the absence of malonate inhibition. It should be noted, however, that the TPN-linked malic enzyme is not inhibited by malonate (Ochoa, 11, p. 747).

A similar phenomenon has been reported by Fuller and Anderson (7), who found that the first stable products of photosynthesis of purple sulfur bacteria were PGA and aspartic acid. They attributed the early appearance of aspartic acid to a light-stimulated carboxylation of phosphoenolpyruvate to oxaloacetate followed by transamination with glutamine to give aspartic acid.

An A.Q. of less than one-half for the initial transient gas exchange as is shown in figure 3 can be accounted for by an establishment of a steady-state concentration of [OH] in the light. The oxidizing power of [OH] may be stored in one or more highly oxidizing compounds involved in the evolution of O_2. When the light is turned on, some of the photochemically produced [OH] is used to establish this concentration of [OH] while the concomitantly produced TPNH is utilized in carboxylation reactions with the result that the A.Q. is less than half until the steady-state concentration of [OH] is established. When illumination ceases the [OH] stored during the light is utilized for oxidative decarboxylations, either directly or via the oxidation of TPNH to TPN, causing the release of CO_2 shown in figure 3 following the cessation of illumination. The hypothesis that a steady-state concentration of [OH] is maintained in the light finds support in the work of Chance and Strehler (3) who show that light has an oxidizing affect on green plants.

The same hypothesis also accounts for the gas exchange exhibited by heat-treated leaves (fig 5) if we assume that the heat treatment has destroyed an enzyme necessary for the evolution of O_2 from [OH]. In the light the concentration of [OH] builds up until it back reacts with [H] or TPNH as rapidly as it is formed. Until the steady-state concentration of [OH] is established TPNH is spared for carboxylation reactions resulting in initial uptake of CO_2. Pyruvate is available for the carboxylation since the heat treatment has not affected the rate of respiration. When the light is turned off the steady-state concen-

tration of [OH] causes decarboxylations equal to the initial carboxylations.

In addition to showing a transient release of CO_2 darkening with heat-treated leaves, Van der Veen also observed it with normal leaves in atmospheres of H_2 with 2 % CO_2 and traces of O_2 particularly at low temperatures. However, since he did not observe this CO_2 gush when an atmosphere of air with 3 % CO_2 was used, he attributed the phenomenon to an inhibitory effect of H_2. This cannot be the case, however, for the same phenomenon is exhibited in He atmospheres. Veljby (15) shows that it also occurs in air if the light is turned off just after the initial uptake of CO_2 while the rate of photosynthetic assimilation is still low. We have also observed it with leaves in air and 4 % CO_2 at 0° C where the steady-state rate of photosynthesis is suppressed. It is generally not so apparent when illumination ceases while the rate of photosynthesis is high because it is masked by the dark-uptake of CO_2 due to the carboxylation of the ribulose diphosphate present when illumination ceased.

Summary

Both O_2 and CO_2 exchange have been measured during the transitory uptake of CO_2 observed at the beginning of the photosynthetic induction period of leaves. The A.Q. during this process is approximately one-third. Experiments made at 0° C in 4 % CO_2 to isolate the transitory carboxylation from normal photosynthesis established the pronounced influence of the duration of the previous dark and light periods. A mechanism based on the light-induced reductive carboxylation of pyruvate to malate is presented to account for the observed phenomena. The transitory release of CO_2 sometimes observed on darkening and the reversible CO_2 exchange of heat-treated leaves are also discussed.

Acknowledgement

I wish to express my appreciation to Professor James Franck for the encouragement and direction which he gave during the course of this work.

Literature Cited

1. Aufdemgarten, H. 1939. Zur Kenntnis der Sagenannten Induktionsvorgänge bei der Kohlensäureassilation. Planta 29: 643–678.
2. Aufdemgarten, H. 1939. Weitare Untersuchungen mit dem Gaswechselschreiber über die Kohlensäure—Assimilation. Planta 30: 343–352.
3. Chance, B. and B. Strehler 1957. Effects of oxygen and red light upon the absorption of visible light in green plants. Plant Physiol. 32: 536–548.
4. Conn, E., B. Vennesland, and L. M. Kraemer 1947. Distribution of a TPN-specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid in higher plants. Arch. Biochem. 23: 179.
5. Emerson, R. and C. M. Lewis 1939. Carbon dioxide exchange and the measurement of the quantum yield of photosynthesis. Amer. Jour. Bot. 26: 808.
6. Franck, J. 1953. Participation of respiratory intermediates in the process of photosynthesis as an explanation of abnormally high quantum yields. Arch. Biochem. Biophys. 45: 190–229.
7. Fuller, R. C. and I. C. Anderson 1957. Co_2 assimilation in photosynthetic purple sulfur bacteria. Plant Physiol. 32 suppl.: xvi.
8. Gaffron, H. 1957. Transients in the CO_2 gas exchange of algae. In: Research in Photosynthesis. Pp. 430–443. Interscience Publisher Inc., New York.
9. Massini, P. 1957. Photosynthetic Phosphorylation as an Explanation for Induction Phenomena in Photosynthesis. Acta Bot. Neerl. 6: 434–444.
10. McAlister, E. D. and J. Myers 1940. The Time Course of Photosynthesis and Fluorescence Observed Simultaneously. Smithsonian Miscellaneous Collections. 99: 1–37.
11. Ochoa, S. 1955. In: Methods in Enzymology. Vol. 1: 739–747. Academic Press, New York.
12. Van der Veen, R. 1949. Induction phenomena in photosynthesis I. Physiol. Plantarum 2: 217–234.
13. Van der Veen, R. 1949. Induction phenomena in photosynthesis II. Physiol. Plantarum 2: 287–296.
14. Veljby, K. 1958. Induction phenomenon in photosynthesis. Physiol. Plantarum 11: 158–169.
15. Veljby, K. 1958. Induction phenomenon and CO_2 gush in photosynthesis of *Polytrichum attenuatum*. Physiol. Plantarum 11: 866–877.

INTERACTION OF ENDOGENOUS AND ENVIRONMENTAL PERIODS IN PLANT GROWTH [1, 2]

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In its natural environment the plant is exposed to alternating periods of light and dark, the combined lengths of which are always 24 hours. Thus we may speak of plants in nature as growing on a 24 hour cycle length. Garner and Allard (2) investigated the effect of light-dark cycles shorter than 24 hours on growth and on flowering. They found that in general such cycles cause reduction in growth. Highkin and Hanson (4) have reported that tomato plants grown under 12 hour and 48 hour cycles become abnormal and that growth is markedly reduced as compared with that of plants grown on a 24 hour cycle. Verkerk (8) has shown that tomatoes require a daily dark period for normal development. Hillman (5) also found that leaf injury appears very quickly when tomatoes are grown in continuous light or in cycle lengths which deviate too greatly from 24 hours, e.g., 18 hours or shorter. Hillman found in addition that the requirement of tomatoes for a daily dark period can be replaced by a period, in light, at a temperature much lower than that during the balance of the cycle. It may be concluded that normal development of the tomato requires that each cycle include a dark period (or a period at low temperature) and that the length of the cycle must be close to 24 hours. This suggests that the plant possesses some endogenously rhythmic process with a period of 24 hours, which is involved in the growth process, and which must be properly in register with the environmental cycle.

Endogenous rhythms are known to participate in and regulate other phases of plant behavior. Thus the leaf movements of beans possess an endogenous rhythmicity. Bünning (1) has shown that the period of such leaf movements of Phaseolus is but slightly temperature dependent. The period, which is 24 hours at 26° C increases to approximately 30 hours at 15° C. A comparable lengthening of the period of the photoperiodic response in the soybean has been found by Nanda and Hamner (6). Plant growth rate is also temperature sensitive (Went, (9)) and decreases in growth rate under unfavorable temperature conditions might be due in principle to effects of temperature on the period of such an internal rhythm. It has in fact been shown by Went (9) that the African Violet (*Saintpaulia ionantha* Wendl.) grows but poorly at 10° C on a 24 hour cycle, but grows more vigorously at the same temperature if the cycle length is increased to 32 hours. Went obtained similar results with *Baeria chrysostoma* Fisch. and Mey. as well as with tomatoes.

The present paper deals with further studies in this field. It will be shown that the environmental cycle length which yields maximal dry matter production becomes longer as the temperature is lowered and the reverse. Symptoms of temperature injury can be lessened or prevented by adjusting the length of the environmental cycle and without alteration of the temperature.

MATERIAL AND METHODS

Tomato seeds, variety Extra Early (*Lycopersicon esculentum* Mill.), and peanut seeds, variety NC2 (*Arachis hypogaea* L.), were planted in a mixture consisting of equal parts (by volume) of crushed rock and Vermiculite and were supplied with Hoagland's solution. The seedlings were raised under a standard 24 hour cycle (16 hours light, 8 hours dark) in a greenhouse at 26° C day temperature (for 8 hours) and 20° C night temperature (for 16 hours), or in artificial light at a constant temperature of 26° C. Two weeks after germination, uniform plants were selected and randomly divided in groups of 15 plants. One group of plants was used for each treatment.

Special cabinets were used to give alternating light-dark cycles to the plants. Four cabinets were installed in each of three similar rooms each maintained at different temperatures. The light for the cabinets consisted of fluorescent tubes (T8, warm white) interspersed with lumiline incandescent strip lights. The whole assembly was hung above and outside the cabinets. Each cabinet had a sliding top which was opened or closed pneumatically. The time of opening and closing each cabinet was regulated by a time clock. Each cabinet held three racks of 15 plants each and the racks were adjusted independently with respect to distance below the light panel.

All cycles consisted of equal periods of light and darkness. Therefore the total amount of light and of darkness received during an experiment was the same for all treatments. When a plant received 12 hours light and 12 hours darkness, the cycle length under which it is grown is 24 hours according to the terminology used in this paper. Light intensity during the experiments was maintained at 750 to 800 ft-c

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TABLE I

DRY MATTER ACCUMULATION BY PEANUT PLANTS AT DIFFERENT TEMPERATURES AND CYCLE LENGTHS

TEMP. °C	CYCLE LENGTH—HRS 20	22	24	27	30	33
14			0.76 ± 0.06	1.35 ± 0.06	0.94 ± 0.04	0.67 ± 0.08
23	1.30 ± 0.10	1.73 ± 0.15	1.53 ± 0.17	1.46 ± 0.08		
30	1.04 ± 0.07	0.77 ± 0.05	0.38 ± 0.02			

The dry matter accumulation is given as the increase in dry weight (grams/plant) during 25 days of growth in the experimental conditions.

at the top of the plants unless otherwise specified. Dry weight (determined ± 5 mg) was used as a measure of plant growth. The cycle lengths were allocated at random to the four cabinets at each temperature.

RESULTS

Table I presents data concerning dry weight accumulation for an experiment with the peanut. At 14° C the optimum cycle length is 27 hours; at 23° C it is 22 hours, and at 30° C it is 20 hours. The experiment of table I did not include cycles shorter than 20 hours, but in other experiments it has been found that the accumulation of dry matter at 30° C decreases when the plants are grown under cycles shorter than 20 hours (see table III). The optimum cycle lengths found were not identical in all experiments (table II).

TABLE II

OPTIMUM CYCLE LENGTH FOR GROWTH OF PEANUT AND TOMATO PLANTS AT DIFFERENT TEMPERATURES

	30° C		23° C		14° C	
PLANT	20	22	22	24	27	30
Peanut	3	0	6	0	5	1
Tomato	3	1	5	1	8	1

The table indicates the number of experiments in which a given cycle length was found to give maximal dry matter production at the temperatures indicated.

The results of the experiments can be summarized as follows: the optimum cycle length is 27 to 30 hours at 14° C; it is 22 hours at 23° C, and 20 hours at 30° C. Variation in optimal cycle length as between different experiments at the same temperature is probably due to the cycle lengths chosen and the magnitude of the difference between successive cycle lengths. The determination of the optimum cycle length is necessarily an approximation.

Table III is a summary of the data obtained. The data given for 14° C and 23° C are averages from six experiments. In the case of 30° C the averages are based on three experiments. For statistical analysis the data have been given as the total increase in dry weight of 15 plants during 25 days of growth under the experimental conditions. Each experiment has been treated as a single replicate. The treatment, in this case the change in length of the light-dark cycle, has a significant effect at all three temperatures used. It can be concluded from table II that the optimal cycle length is quite reproducible between experiments.

Peanut plants grown under a 24 hour cycle showed obvious signs of injury in both high and low temperature conditions. The leaves produced in the high temperature regime were small with necrotic spots and yellow tips. Such injury was absent in plants grown at the same temperature but under a 20 hour cycle. Temperatures lower than 14° C during the light period and with a 24 hour cycle caused marked chlorosis. Apparently the rate of chlorophyll synthesis could not keep pace with the rate of leaf growth. The youngest leaves therefore were yellow although

TABLE III

EFFECTS OF TEMPERATURE AND CYCLE LENGTH ON DRY MATTER PRODUCTION BY PEANUT PLANTS

TEMP. °C	CYCLE LENGTH—HRS 18	20	22	24	27	30	33	
14 ± 0.5				7.11	10.93	9.49	8.90	*
23 ± 0.3		18.83	23.09	18.59	18.26			**
30 ± 0.7	12.64	16.47	14.62	12.80				***

Data are expressed as total increase in dry weight (in g) of 15 plants after 25 days in the experimental conditions.

* Means of six experiments. Smallest significant difference at $P<0.01$ is 1.3 g.
** Means of six experiments. Smallest significant difference at $P<0.01$ is 1.1 g.
*** Means of three experiments. Smallest significant difference at $P<0.01$ is 2.4 g.

they became green as they grew older. This injury was absent when the plants were grown under a 27 hour cycle. It is clear that the loss in dry weight due to unfavorable temperature was reduced by adjusting the external cycle to that optimal for a particular temperature. In individual experiments large differences in dry matter accumulation were observed when the cycle length was adjusted (table I). Differences in appearance of the plants were very marked.

The data presented (tables I, III) also show that the magnitude of the effect is not the same at all temperatures. The relative effect of cycle length is larger at the extreme temperatures than at the optimum temperature. Apparently unfavorable temperature makes the plants more sensitive to variations in cycle length.

Results comparable to those obtained with the peanut have also been obtained with the tomato. In the tomato plant, in contrast to the peanut, however, no visible leaf damage resulted from unfavorable temperature. The optimum cycle lengths for dry matter accumulation were closely similar to those for the peanut (table II).

In the above experiments seeds were germinated and grown under a 24 hour cycle before they were exposed to other cycle lengths. Similar results were obtained when the plants were grown from seed under the experimental conditions. Data from such an experiment are given in table IV which includes also data for control plants raised in the normal manner (2 weeks on a 24 hour cycle before transfer to varied cycle lengths). The data of table IV show that the results were not qualitatively changed by germinating the plants under the experimental conditions.

TABLE IV

EFFECT OF CONDITIONS DURING GERMINATION ON SUBSEQUENT DRY MATTER PRODUCTION OF TOMATO PLANTS

	Cycle length—Hrs				
	24	27	30	33	
I	0.86	1.40	1.18	1.10	*
II	0.47	1.00	0.75	0.65	**

Dry matter production has been expressed as the increase in dry weight in g/plant. The data are means of two experiments. Temperature, 14° C.

I. Effect of cycle length on increase in dry weight of plants raised in the standard manner (see methods).

II. Effect of cycle length on increase in dry weight of plants raised from seed under the experimental conditions.

* Smallest difference for significance at $P<0.01$ is 0.3 g.

** Smallest difference for significance at $P<0.01$ is 0.27 g.

DISCUSSION

Results of the present experiments are in agreement with those of Went on Saintpaulia (African Violet), *Baeria chrysostoma*, and tomato (unpublished data). For maximal development all of these plants require an alternating light-dark cycle with a cycle length close to 24 hours. The optimal cycle length, however, is dependent on the prevailing temperature. The amount by which the optimal cycle length changes with temperature approximates the amount by which the periods of autonomous diurnal rhythms change with temperature. Bünning (1) has shown that the period of leaf movement in beans is decreased by 7.7 hours when the temperature is raised from 15 to 30° C. The results suggest then that plants possess some endogenous, cyclical time-measuring device and that for optimal growth the external period must be synchronized with the endogenous period of the plant. The endogenous clock appears to run more slowly at lower temperature and more rapidly at higher temperature. At such temperatures it is not therefore in register with an environmental 24 hour cycle. Lack of synchronization between the endogenous and the environmental light-dark cycle is apparently the cause of a part of the reduction of growth observed in plants grown under unfavorable temperature conditions. Relatively small changes in cycle length have a significant effect on plant growth and development.

It is of further interest that unfavorable temperature increases the sensitivity of plants to changes in the environmental periodicity. Conditions which weaken the plants apparently cause them to be more sensitive to environmental cycle length.

The mechanism by which cycle length affects elongation and dry matter production in the plants studied is not yet known. There have, however, been reports of periodicity in metabolic processes in plants. Thus Griesel and Biale (3) have shown that respiration of petals of *Magnolia grandiflora* exhibits rhythmicity. The same has been found by Richter and Pirson (7) for the activity of acid phosphatases and catalase in Hydrodictyon. It is conceivable that such rhythmic processes might suffer if synchrony between the endogenous and environmental periods were not maintained. Reductions in growth might possibly be caused in some such manner.

SUMMARY

It has been shown that the reductions of growth of peanut and tomato plants caused by unfavorable temperature can be prevented to a certain extent by adjusting the length of the light-dark cycle under which the plants are grown. The cycle length which produces optimal growth is 22 hours at 23° C, 27 to 30 hours at 14° C, and 20 hours at 30° C. Environmental conditions which weaken the plant increase the response to changes in length of the environmental cycle.

It has been concluded that peanut and tomato possess an endogenous, time-measuring mechanism, which is slightly temperature dependent, and that for optimal development the external period must be synchronized with the endogenous period of the plant.

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Literature Cited

1. Bünning, E. 1931. Untersuchungen über die autonomen tagesperiodischen Bewegungen der Primärblätter von Phaseolus multiflorus. Jahrb. wiss. Bot. 75: 439–480.
2. Garner, W. W. and H. A. Allard 1931. Effect of abnormally long and short alterations of light and darkness on growth and development of plants. Jour. Agr. Res. 42: 629–651.
3. Griesel, W. O. and J. B. Biale 1958. Respiratory trends in perianth segments of *Magnolia grandiflora*. Amer. Jour. Bot. 45: 660–663.
4. Highkin, H. R. and J. B. Hanson 1954. Possible interaction between light-dark cycles and endogenous daily rhythms on the growth of tomato plants. Plant Physiol. 29: 301–302.
5. Hillman, W. S. 1956. Injury of tomato plants by continuous light and unfavorable photoperiodic cycles. Amer. Jour. Bot. 43: 89–96.
6. Nanda, K. K. and K. C. Hamner 1959. The effect of temperature, auxins, antiauxins, and some other chemicals on the endogenous rhythm affecting photoperiodic response of Biloxi soybean (*Glycine max*. L.; Merr.). Planta 53: 53–68.
7. Richter, G. and A. Pirson 1957. Enzyme von Hydrodictyon und ihre Beeinflussung durch Beleuchtungsperiodik. Flora 144: 562–597.
8. Verkerk, K. 1955. Temperature, light, and the tomato. Mededel. Landbouwhogeschool Wageningen 55: 176–224.
9. Went, F. W. 1957. The Experimental Control of Plant Growth. Chronica Botanica Co., Waltham.

RELATIONSHIP OF GROWTH AND DEVELOPMENT TO CHANGES IN SUGARS, AUXINS, AND GIBBERELLINS IN FRUIT OF SEEDED AND SEEDLESS VARIETIES OF VITIS VINIFERA [1, 2]

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There are large differences in the degree of seed development in different varieties of *Vitis vinifera* (European grape) ranging from nil in parthenocarpic varieties to complete seed development from several of the four ovules in the berries of certain other varieties. A few varieties are "seedless" because of their stenospermocarpic fruit development, i.e., their seeds begin to develop after fertilization but they abort before the development is complete. These differences in seed development are reflected in growth of the berries. This study was made to relate the development and growth of the fruit of several varieties to changes in their sugar, auxin, and gibberellin contents between anthesis and maturity.

The morphological changes in fruit of a few seeded and seedless varities of grapes have been described (16, 20, 24). Fruit morphogenesis was measured in a semi-quantitative way in the varieties used here to help interpret the other factors measured. Fruit growth studies of grapes are not extensive. Most have the disadvantage that they are based on fruit diameter measurements (33). In this study fresh weight, dry weight and volume of berries, and sugar concentration in the juice were measured.

The most detailed work on auxins in grapes has been done by Nitsch and coworkers. They demonstrated the presence of several types of auxins in nearly mature grapes (14) and made a comparative study of auxin changes during the development of berries of seeded and seedless Concord (16). Gustafson's comparison of auxins in seeded and seedless grapes was made on flowers before anthesis (6). Ferenczy (5) showed the presence of an auxin, possibly IAA[4], in the juice of ripe grapes. The measurement of gibberellin activity was attempted because of

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[4] Abbreviations used are: IAA, 3-indoleacetic acid; IAN, 3-indoleacetonitrile; IAE, ethyl ester of IAA; 4-CPA, 4-chlorophenoxyacetic acid; GA_3, gibberellin A_3 (synonymous with gibberellic acid), similarly for GA_1 and GA_2.

the recently appreciated importance of these compounds as plant growth hormones (22) and the profound effects of exogenous GA_3 on the growth of seedless grapes (28).

Materials and Methods

Five varieties of grapes were chosen to give three types of seed development: A. Perfect (Muscat of Alexandria and Emperor), B. Stenospermocarpic (Seedless Emperor and Sultanina) and C. Parthenocarpic (Black Corinth). The two Emperor varieties mature later than the other varieties. The development of young fruit of Muscat and Sultanina was followed by microscopic examination of longitudinal and transverse sections (prepared in 1934 by Prof. H. P. Olmo) of ovaries taken at intervals from 10 days before to 23 days after anthesis. Measurements were made of dimensions and cell size within different seed and fruit structures to estimate changes in the rate of cell division. For example, counts were made of the number of cells along minimal distances across the pericarp; this was done in five positions on each of six berries at each stage. For Muscat, this number doubled between the third and tenth days after anthesis and during this time many new cross walls were evident. These observations were supplemented by dissection of preserved and lyophilized berries. Few measurements were made of embryos because they were difficult to dissect.

Ten vines of each variety were selected in the university vineyard at Davis during the summer of 1958. In addition, ten vines each of Corinth and Sultanina were trunk-girdled at the end of bloom and 10 days after bloom, respectively. Girdling consisted of removing a complete strip of bark (5 mm wide) down to the cambium after making two parallel knife cuts around the trunk. From these seven lots of vines, flower or fruit samples were taken every 3 to 5 days from the beginning of bloom until maturity. Each sample was a composite of several berries from many clusters on all vines. In all, no more than a tenth of each vine's crop was removed. A sub-sample of 200 berries (gradually decreasing to 50 berries as they enlarged) was weighed, its volume determined by water displacement, then dried at 90° C to measure dry weight. Berries reaching maturity dried very slowly. A second sub-sample was crushed and the percentage of sugars in the free-run juice measured with an Abbé refractometer. This instrument is calibrated against sucrose solutions but it gives a close approximation to the amount of total soluble solids, mainly glucose and fructose, in grape juice. Excluding sampling error, the error in measuring fresh weight was low ($\pm 0.2\%$) but that of volume was high (± 1 to 2%). To help follow the changes in volume, values for density (weight/volume) of each variety were graphed and a smoothed curve drawn (the density remained between 1.00 and 1.01 g/ml until the beginning of sugaring, when it increased gradually to 1.08-1.10 g/ml); volumes were recalculated from the densities read from these curves and from the fresh weights. The size of the sampling error can be gauged by the distance of points from the weight curves shown for Muscat (fig 3). Other varieties behaved similarly so their fresh weight curves are omitted and only smoothed volume curves are shown.

On 16 of the 25 sampling occasions, about one liter of berries was frozen shortly after collecting, held at −12° C and later lyophilized. Samples collected 17 days after anthesis, and later, failed to dry during 2 days lyophilization unless the skins of the berries were cracked; this was done by striking the plastic bag of frozen berries against a bench. A few samples collected between 17 and 25 days after anthesis spoiled because they were not dried completely.

Extraction and Measurement of Auxins. Preliminary comparisons of extraction and purification methods led to adopting a procedure similar to that used by Nitsch (13). A weighed sample of lyophilized berries (1-1.5 g dry wt) was ground and extracted with 50 ml methanol at 4° C for 3 to 4 hours. The filtrate was dried under an airstream and the residue extracted four times with acetonitrile and hexane (1:1) in a separatory funnel. After shaking, the clear yellow acetonitrile solution was run off and divided into two or three equal parts (in case repeats were needed), then dried under an airstream and stored at −12° C. Three hours before bioassay, a measured volume of sucrose-buffer solution (15) was added to the residue to give a desired concentration of grape extract. After 1 hour, this was filtered and 0.5 ml aliquots of the filtrate (or dilutions of it) were used for the bioassay.

Auxin was measured by the method of Nitsch and Nitsch (15) using straight growth of sections of Avena first internodes. Their method was modified as follows: seed was grown in vermiculite for 60 hours at 26° C; only two sections were placed in each tube containing 0.5 ml sucrose-buffer-auxin solution and each treatment was replicated four times (26); the tubes were rotated at 0.2 rpm in a roller tube apparatus. A control without IAA and five solutions containing IAA at concentrations between 0.001 and 0.1 μg per 0.5 ml were run with every test.

For each extract, three dilutions were assayed (or two in some repeated runs). Solutions from some extracts with high auxin concentration had to be diluted to as little as 5 mg of berries (dry wt) per 0.5 ml to fall within the test's sensitivity. The strength of solutions from extracts with low auxin concentration was increased to 24 mg per 0.5 ml. Some strong solutions of extract were toxic to the internode sections causing them to become flaccid and reducing their elongation. Bentley (1) has also described this. The trouble from phytotoxicity was confined to extracts of berries sampled later than 14 days after anthesis and ceased when sugaring began. Usually only slight dilutions were needed to remove phytotoxicity and reveal auxin activity. In each run, from 8 to 12 extracts were compared at two or three dilutions of each. Each sample of lyophil-

ized grapes was extracted and assayed two or three times.

The dilutions of grape extracts showed a steeper dosage-response curve than IAA. The average ratio of the slope of the grape extract response curve to the IAA curve was about two. This difference could be due to the presence of inhibitors or of auxins other than IAA. Nitsch and Nitsch (14) have shown that grapes probably contain IAN and IAE as well as IAA. They later (15) showed that the standard curves for IAN and IAE are steeper than that for IAA. Because of this difference in slope the IAA standard curve could not be used to compare results, so the following arbitrary procedure was used: A new standard curve was prepared, double the slope of the IAA curve and intersecting the latter at its midpoint, viz. 0.01 μg IAA per 0.5 ml (this reduced high values and increased low values). From this curve, internode elongation was corrected to "μg—equivalents of auxin". These were adjusted to "μg—equivalents of auxin per gram dry wt. of grapes" and these are called "auxin units".

Extraction and Measurement of Gibberellins. The terms "gibberellin" and "gibberellin activity" are used to include known gibberellins and other gibberellin-like compounds which have the property of causing elongation of seedlings of the dwarf mutants of maize used in the bioassay. Measurements of gibberellin activity were made on all samples which were assayed for auxin content, and, in addition, flowers at anthesis were assayed after separation into ovaries and stamens. The following extraction procedure was used (Phinney, personal communication): a lyophilized sample was weighed (1-8 g dry wt of ovaries, less for stamens), ground with a pestle and mortar, extracted with 30 to 50 ml acetone for 3 hours at room temperature, then filtered; the filtrate was dried under an airstream, extracted with ethyl acetate in the presence of excess anhydrous sodium sulfate to remove water, then filtered; the filtrate was dried under an airstream and stored at −12° C until an hour or two before its bioassay when a measured volume (3-10 ml) of water plus Tween 20 (polyoxyethylene sorbitan monolaurate, 0.05% v/v) was added. After filtering, 0.1 ml aliquots were applied to each test plant.

The amount of gibberellin in these extracts was measured by Phinney's dwarf maize bioassay (11; Phinney, personal communication). The dwarf mutants d-1 and d-5 were used for these assays (seed kindly supplied by Prof. B. O. Phinney); d-1 was used for the first test of all samples, then, when retesting active samples, comparisons were made with d-5. Unknown extracts, a control solution and standard solutions containing from 0.001 to 0.2 μg GA_3 per 0.1 ml were combined in randomized complete block designs with 4 to 8 replicates. Seven days after treatment the lengths of the first and second leaf sheaths were measured in mm and added (=D). The average of the values for control seedlings (D_o) was subtracted from the individual values (D_x) for control and treated seedlings to give a value for elongation ($E_x = D_x - D_o$). Results were compared by analysis of variance after transformation to log ($E_x + 10$). This transformation gave a straight line relation to concentration of GA_3 on a log scale between 0.001 and 0.2 μg GA_3 per plant and it increased the sensitivity of comparisons at low concentrations.

This study of the changes in the levels of auxins and gibberellins during the development of grapes should be considered as exploratory. Only crude extracts were made and, for gibberellin assays, dilution curves of these extracts were not made. More experiments are needed using berries sampled at more frequent intervals, extracted by several methods, purified by chromatography and tested with more than one bioassay method.

Results and Discussion

Fruit Development. The gross changes in seed and fruit structures during the development of Muscat are shown in figure 1. An estimate of the rate of cell division in different structures of the fruit of Muscat and Sultanina is shown in figure 2.

The development of a Muscat berry is probably typical of all seeded grapes apart from varietal differences in dimensions. It can be considered in two cycles:

Cycle I (0-45 days after anthesis): The growth rates of most parts of the seed and fruit rise to a maximum and then fall. The ratio of seed width and pericarp width remains nearly constant, i.e., both grow at an approximately equal rate. Growth is due to both cell division and cell enlargement. The radial diameters of cells of the outer integument and nucellus increase about three-fold and those of the pericarp and septum about six-fold. These four structures comprise the bulk of the seed and fruit until the endosperm takes the place of the nucellus. Meristematic activity in the outer integument is high; the rate of cell division increases to a maximum at 20 to 25 days then declines to nil at 45 days. The three layers of cells of the inner integument divide anticlinally to accommodate the large size and shape changes of the outer integument. Cell division in the nucellus proceeds at a rate comparable to that in the outer integument but it declines earlier as the endosperm nuclei proliferate. Meristematic activity in the endosperm reaches a peak at 35 days. Only a few divisions occur in the zygote until 25 days after anthesis. Most cell divisions in the pericarp occur between the fifth and tenth days after anthesis (divisions occur also 5-10 days before anthesis). The skin, with four to eight layers of cells, is distinguishable at 16 days and is well defined by 23 days. Meristematic activity in the septum continues throughout cycle I. By 45 days, the seeds are nearly full-sized, but the berry is hard and green and only a third of its mature volume.

Cycle II (45 days - 110 days after anthesis): Berry volume triples and the berry softens and matures. Pericarp width increases two to three times

and all of this increase is due to cell enlargement in the pericarp and septum; there are no cell divisions. Presumably, the embryo continues to grow.

Sultanina berries show similar changes to Muscat except that seed development is imperfect. Pearson (20) has described the excessive growth of the tips of the inner integument which can be seen at anthesis. Olmo's sections show that these abnormalities are evident 11 days before anthesis. The outer integument and nucellus commence growth with rapid cell divisions. The rate of cell division in these parts reaches a maximum 15 days after anthesis and stops by about 30 days. Endosperm nuclei commence divisions but usually, after 20 days, endosperm and nucellus cells start to degenerate. In the mature berry, the stenospermic seed is a slender, soft structure. In one mature seed an undegenerate nucellus was found.

Black Corinth berries grow parthenocarpically.

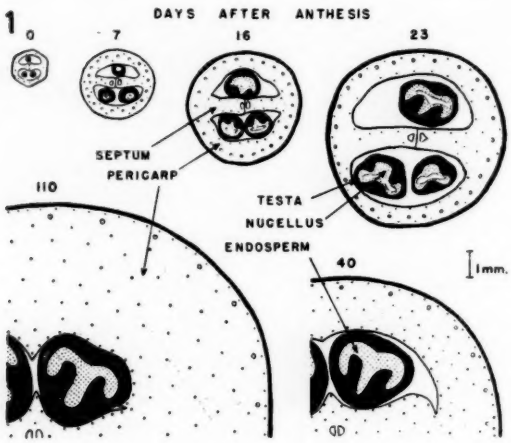


FIG. 1. Diagrams of transverse sections of berries of Muscat of Alexandria at varying times between anthesis and maturity.

FIG. 2. Diagram of changes in rate of cell division in different seed and fruit structures of Sultanina and Muscat. Compiled from sections prepared by Prof. H. P. Olmo. Diagram design after Wright (34).

This is due to defective embryo sac formation and not to defects in any other tissue of the ovule (20). In most berries, the ovule does not develop beyond anthesis; if it does, it develops a hard testa and the berry enlarges. The stimulus of pollination and girdling are needed for good set (17); this is defined by Stout (24) as stimulative parthenocarpy.

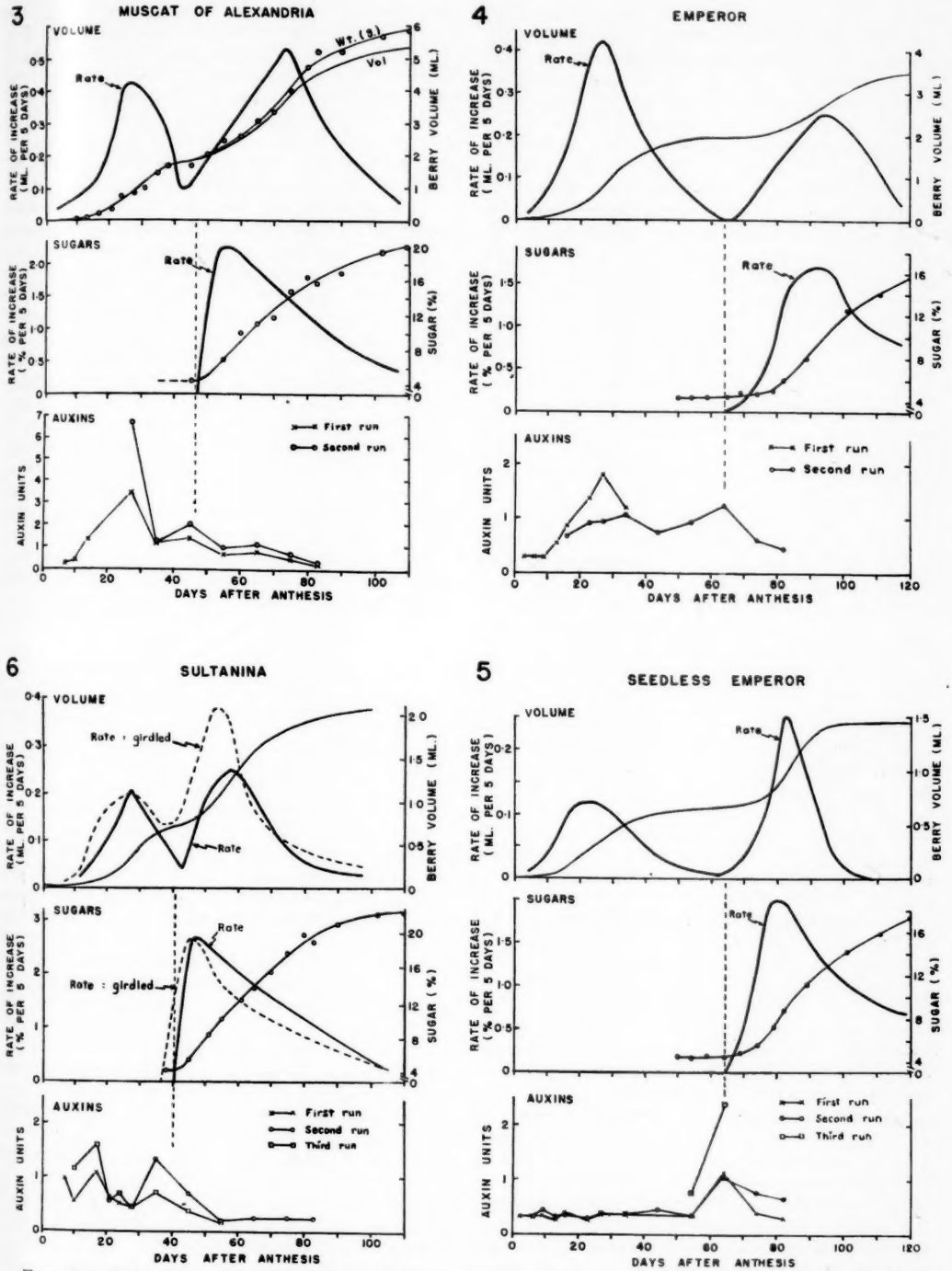
Seedless Emperor berries are stenospermocarpic. Their seed development is very incomplete and variable ranging from none at all to some larger than the stenospermic seeds of Sultanina. No hard testas were found. There is a correspondingly large variation in berry size because the growth of each berry is proportional to the amount of seed growth within. Most berries measure from 9 by 11 to 12 by 15 mm and, in these, seeds rarely lengthen to more than 1.5 mm (c.f. Sultanina seeds which average 3 mm in length and Muscat seeds, 6-7 mm). A few berries enlarge to 16 by 20 mm and some are as small as 5 by 5 mm. Olmo (18) found from one to four small rudimentary seeds in about 70 percent of the berries and, in the rest, no ovule development occurred after anthesis. Considering the slight seed development, it is remarkable that Seedless Emperor berries grow to about 1.5 ml volume compared with 0.25 ml in Corinth.

FRUIT GROWTH AND CHANGES IN SUGAR. The changes in berry volume and the rate of increase in volume from anthesis to maturity are shown for the five varieties in the top graphs of figures 3 to 7. Similarly, changes in sugar concentration in the juice are shown in the middle graph. The changes in berry volume and sugar concentration brought about by girdling of Sultanina and Corinth are shown by their rate curves.

All varieties show a double-sigmoid growth curve and hence a two-humped rate curve. These humps correspond to cycles I and II mentioned above; similarly shaped growth curves of many fleshy fruits have been commonly described in three periods or stages (see review by Nitsch, 12). The late-maturing varieties, Emperor and Seedless Emperor, show this feature clearly. It is barely perceptible in Corinth because the first growth cycle is small. Winkler and Williams (33) concluded that their measurements of berry diameter of Sultanina did not show a double-sigmoid growth curve (although their graph suggests it); the volume measurements reported here show it clearly.

Sugar concentration in the berries of Muscat, Sultanina, and Corinth rises suddenly, almost on a certain day, and the increase in sugar content reaches its maximum rate within ten days. In the later-maturing Emperors, sugaring begins slowly for 5 to 10 days, then rises to a rapid rate. The maximum rate of sugaring for Corinth is nearly double the maximum rate for the other varieties, probably because of its small size. The increase in sugar concentration of the juice of grapes is remarkable (it changes from 4-20 percent in 30 or 40 days).

A comparison of the sugar and growth rate curves reveals the striking coincidence of the commencement



FIGS. 3, 4, 5, 6. Changes in berry volume, juice sugar concentration, and auxin concentration in berries of Muscat, Emperor, Seedless Emperor and Sultanina from anthesis to maturity. Volume and sugar measurements are expressed as cumulative and rate curves; for girdled Sultanina, only the rate curves are shown. Note that ordinate scales differ for each variety. The term "auxin units" is defined in the text.

of sugaring and the second growth cycle. Corinth shows a slight discrepancy but, having smallest berries, this has the greatest percentage error. Both sugaring and growth rate curves increase sharply and, in each variety, the maximum rate of sugaring is reached a few days before the maximum growth rate. It is possible that the second growth cycle of grape berries could be caused by the influx of sugar into the berry. The changes in auxins and gibberellins (discussed below) and the morphological changes do not suggest other mechanisms to explain why the cells of the pericarp and septum enlarge as they do during this stage. From 80 to 90 percent of the total soluble solids in grape juice are D-glucose and D-fructose in about equal amounts; a 20 % solution of glucose has a theoretical osmotic pressure of about 27 atmospheres. Thomas, Ranson, and Richardson (25, p. 121) quote an osmotic pressure of 40 atmospheres measured in grapes. It is suggested that as sugar moves into the cells of the flesh, water also moves in to adjust diffusion pressure deficits. The berries contain some auxins, probably in sufficient amounts to permit stretching of the cell walls. The literature on changes in sugar and fruit volume in the developing figure (3) and apricot (4) suggests that this hypothesis should be examined in studies on the development of other sugary, fleshy fruits.

Nitsch (12) and others have suggested that the lowered fruit growth rate midway between anthesis and maturity may be due to competition between the fruit and the embryo which is developing during this period. This hypothesis cannot be applied to seedless grapes because there is no embryo development.

Changes in Auxins During Fruit Development. Changes in the level of auxins are shown in the bottom graphs of figures 3 to 7. In all varieties except Seedless Emperor, auxins rise from a low level just after anthesis to a high level during the first few weeks. In the two seeded varieties, Muscat and Emperor, this level is higher and maintained for a longer period than in the seedless varieties Sultanina and Corinth. Nitsch et al (16) have shown a similar difference between seeded and seedless Concord and have related the changes to differences in meristematic activity in the seed. The same could be said for Muscat and Sultanina; meristematic activity in the seed of Muscat is maintained about 10 days longer than Sultanina and, similarly, auxin does not decline to a low level until 35 days after anthesis in Muscat compared with 20 days after in Sultanina. In Emperor, which has big seeds, auxin does not reach a low level until 40 days after anthesis. There is no rise in auxin during cycle I in Seedless Emperor. Corinth provides an exception to this correlation between meristematic activity and auxin production because it has a brief but significant flush of auxin at 11 days after anthesis when its ovule is static; no explanation can be given for this.

In all varieties except Corinth there is a second, smaller rise in auxin concentration reaching a maximum at about the time that the second growth cycle and sugaring begin. It is difficult to explain, on morphological grounds, why auxin should rise as it does at this stage. Wright (34) was able to correlate a second rise in auxin level of Ribes nigrum fruit with meristematic activity in the embryo. This cannot be done in seedless grapes because there is no embryo development. Perhaps the rise in auxin just before sugaring, if real, has some causal relationship with sugaring. Some related facts are that exogenous auxins affect the concentration of sugars in plant tissues (7) and also some workers consider that auxin acts as a trigger in affecting plant metabolism (19). It is interesting that defoliation did not delay the onset of the second growth cycle (33). The mechanism whereby sugars accumulate in grapes and other fruits, apparently against a concentration gradient, remains one of the puzzles of plant physiology. The key to sugaring is probably held by the berries themselves.

With the increase in berry size during cycle II, the auxin concentration, on a dry weight basis, declines to a low level. Recalculated to the amount of auxins per berry, the same general changes are apparent except that, at about 20 days after the beginning of cycle II, auxins show yet another rise: this is probably too late to enter into causal considerations of growth during this cycle. Changes in the dry weights of berries of the five varieties are shown in table I so that auxin (and gibberellin) figures may be converted to amounts per berry.

Changes in Gibberellins During Fruit Development. Results of the gibberellin bioassays are shown in figure 8 and table II. Duplicates showed high variability. It must be emphasized that the absence of significant activity in these tests does not mean that gibberellins were absent. Better purification might have revealed smaller amounts or removed masking agents.

The results show that the ovaries of the seeded varieties, Muscat and Emperor, have no significant activity at any stage tested but that their stamens have activity at anthesis. On the other hand the ovaries of the three seedless varieties, especially Seedless Emperor, show activity during the first 14 days after anthesis, but not subsequently. Also, like seeded varieties, their stamens show activity at anthesis. The

Table I
Dry Weights of Berries of 5 Grape Varieties at Certain Intervals After Anthesis

Variety	Dry weight per berry (mg)					
	10	30	50	70	90	Mature*
	Days after anthesis					
Muscat	6	78	170	450	860	1,300
Emperor	3	77	137	150	250	800
Seedless Emperor	3	30	41	45	145	350
Sultanina	4	35	80	270	470	500
Corinth	3	5	11	36	57	60

* Dry weight estimated by slight extrapolation.

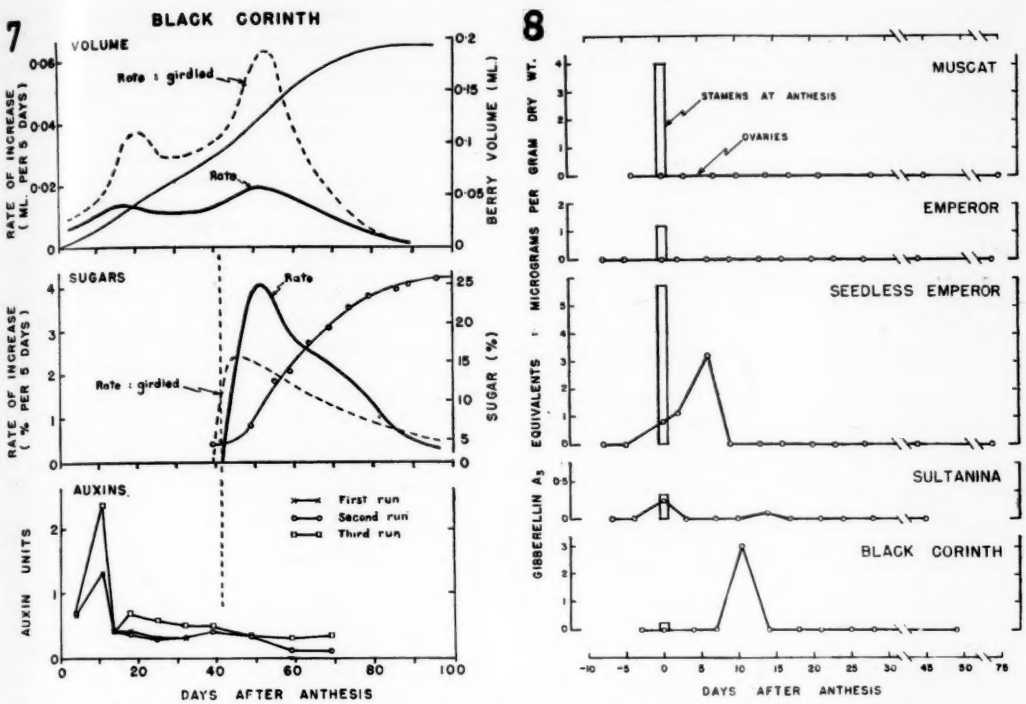


FIG. 7. Changes in berry volume, juice sugar concentration, and auxin concentration in berries of Black Corinth from anthesis to maturity. Volume and sugar measurements are expressed as cumulative and rate curves; for girdled Corinth, only the rate curves are shown. The term "auxin units" is defined in the text.

FIG. 8. The gibberellin activity of extracts of grape stamens and ovaries of different age and variety as measured by the d-1 maize bioassay; note the different ordinate scale for Sultanina.

results suggest also that there may be activity in the ovaries of seedless varieties at anthesis. A fourth seedless variety (Olmo's hybrid No. Q25-6) was tested in June, 1959, and found to have significant gibberellin activity in its young berries, although less than Seedless Emperor.

It may be significant that the difference in gibberellin activity between seedless and seeded berries is correlated with their responsiveness to exogenous GA_3. Without exception, the berries of seedless grape varieties have been found to enlarge following treatment with GA_3 at, or soon after, anthesis, e.g., Corinth and Sultanina (28), Black Monukka and Beauty Seedless (Weaver, private communication), Concord Seedless (23), Seedless Emperor and Olmo's hybrid No. Q25-6 (the author, unpublished data). On the other hand, the berries of seeded grape varieties will not enlarge appreciably after treatment with GA_3 (23, 28). These results suggest that gibberellins may be involved in the growth mechanism during cycle I of seedless berries and that the level of naturally-occurring gibberellins may be an important factor limiting their growth. Gibberellins may not be involved in the growth mechanism of seeded grapes or they are destroyed. More information is needed before these questions can be decided.

The interactions between naturally-occurring auxins and gibberellins of seedless grapes are interesting. Seedless Emperor has low auxin content during cycle I yet it grows five times bigger than Corinth. Perhaps this is explained by the high gibberellin activity in its young berries. This idea is supported by the fact that Corinth berries, when dipped in GA_3 solution (500 ppm) at anthesis (28), enlarge to about the same size as, and ellipsoidal shape of, Seedless Emperor berries even though their ovules remain minute. Thus it is possible that natural levels of gibberellins may be the most important factor limiting their size. Girdling and 4-CPA are not as potent as GA_3 in enlarging Corinth berries.

Comparisons of the activity of grape extracts in elongating d–1 and d–5 maize seedlings are shown in table II. It can be seen that, in most cases, both the young berries of seedless varieties and mature stamens of seeded and seedless varieties induce greater elongation in d–5 than in d–1. Obviously, it would have been better to use d–5 to examine changes in developing grapes rather than d–1. A similar differ-

TABLE II

COMPARISON OF ACTIVITY OF GRAPE EXTRACTS IN ELONGATING DWARF-1 AND DWARF-5 MAIZE SEEDLINGS †

EXTRACT		ELONGATION OF 1ST AND 2ND LEAF SHEATHS MM		GA_3 EQUIVS./ 0.1 ML EXTRACT G × 10^{-9}	
VARIETY	PART	d-1	d-5	d-1	d-5
Gold (seeded)	Stamens	2.0	11.6**	0	1.5
Muscat Seedless	Stamens	11.0**	34.9***	2.2	50
	Stamens	14.3***	22.7***	4	8.3
Emperor	Ovaries††	9.5**	12.9**	1.6	1.8
Sultanina	Stamens	2.8	21.6***	0	9
	Ovaries	3.6	18.0***	0	7
	Pedicels	3.2	4.8	0	1
Corinth	Flowers	1.6	6.9*	0	3

† 0.1 ml of extract (containing from 1-11 mg dry wt of the original grape sample) was applied to each maize seedling. Four or five replicates of pairs of dwarfs were used. Elongations which were significantly greater than control in the analyses of variance are marked by 1, 2, or 3 asterisks when the probabilities that they were significantly greater than controls were better than 20 : 1, 100 : 1 and 1000 : 1, respectively; insignificant values are called nil.

†† Sampled 7 days after anthesis; all other parts in this table were taken at anthesis.

ence in extracts of bean seeds has been found by Phinney and Neely (21) and West and Murashige (30). They purified two crystalline compounds from bean seed. Bean factor I behaved identically with GA_1. Bean factor II was ten times as active on dwarfs -2, -3, -5 and an-1 compared with its activity on d-1. It also differed in its chromatographic behaviour, infra-red spectrum and chemical properties from GA_3, GA_1, and GA_2. Perhaps the activity in grapes is due in part to the presence of bean factor II or other unknown gibberellin-like compounds.

EFFECTS OF GIRDLING ON BERRY GROWTH AND ON CONTENT OF SUGARS, AUXINS, AND GIBBERELLINS. It is common viticultural practice to girdle the trunks or canes of Corinth vines at anthesis to increase fruit set and size and to girdle Sultanina 10 days after anthesis to produce large berries for table use (8). Its effects on berry weight and volume (figs 6 and 7) are apparent within a few days of treatment and these differences continue to increase until maturity. Thus girdling affects both growth cycles. The second growth cycle and sugaring began concurrently a few days earlier in girdled vines of both varieties than in ungirdled. This supports the hypothesis that sugar influx may cause the second growth cycle. The rate of sugar increase in berries on ungirdled vines eventually exceeds that on girdled vines probably because of the bigger crop on the latter.

The effects of girdling on auxin content of the berries are shown in table III. The differences associated with girdling are very small and are far exceeded by variations between tests. The results suggest that the concentration of auxins on a dry weight basis is unchanged. If this is true, the amount per unit volume or fresh weight would also be equal as percentage dry weight and densities are equal in girdled and ungirdled berries at these growth stages. However, the amount per berry would be greater by a factor of 1.5 to 2 in girdled berries because of their greater size. Measurements of gibberellin activity in these samples are too few and too variable to be worth presenting. In four instances, there did not seem to be any large difference on a dry weight basis.

There are several considerations which place importance on the events taking place in the berries of Sultanina during the period from 10 to 15 days after anthesis: the effect of girdling and berry thinning in increasing berry size is maximal (31, 32); treatment with 4-CPA and GA_3 causes greatest enlargement of berries when applied during this period (27, 28); gibberellin activity can be detected in the berries (fig 8) and auxin concentration is high (fig 6). The fact that the rate of cell division in the pericarp is also maximal (fig 2) suggests that girdling and other treatments may increase berry size by increasing the number of cells. The possibility that cell size is also increased should not be excluded.

The effects of girdling on berry size of Sultanina can be duplicated by treatment with low concentrations of 4-CPA and GA_3, so the mechanism of the action of girdling may be due to its effect on naturally-occurring growth regulators. The behavior of seeded varieties supports this idea and emphasizes the

TABLE III

AUXIN CONCENTRATION IN BERRIES OF VARIOUS AGES FROM GIRDLED AND UNGIRDLED CORINTH AND SULTANINA VINES*

EXTRACT			AUXIN UNITS** EXPRESSED ON DRY WT BASIS	
VARIETY	DAYS AFTER ANTHESIS	TEST NO.	UNGIRDLED	GIRDLED
Corinth	14	A	0.37	0.36
		B	0.42	0.34
		C	0.43	0.56
	32	A	0.30	0.32
		B	0.33	0.38
		C	0.51	0.37
	49	A	0.36	0.30
Sultanina	17	A	1.08	1.02
		B	1.59	1.92
	24	A	0.51	0.54
		B	0.72	0.61
	28	A	0.45	0.55
		B	0.45	0.51
	55	A	0.20	0.19
		B	0.16	0.15
	75	A	0.25	0.19

* Equal dry weights of girdled and ungirdled berries were compared in each bioassay.

** The term "auxin units" is described in the text.

probably important role of gibberellin; their berries are devoid of (or low in) gibberellin and they do not enlarge much after girdling or spraying with 4-CPA or GA_3. However, girdling affects the concentration of many compounds, e.g., it increases the level of carbohydrates (29) and decreases the level of nitrate-nitrogen in petioles opposite the grape clusters (9). More information is needed before a conclusion can be made on the mechanism of the girdling effect.

General Conclusions

Correlations between growth rate, auxins and meristematic activity have been demonstrated in several fruits and other plant organs. There are examples in fruits, however, where auxin increase can be correlated with increased meristematic activity in the seed (especially the endosperm) but this activity cannot always be correlated with increased growth rate in the fruit, e.g. apple (10) and figure 2. Failure to show a correlation may be due to failure to measure the substances responsible for growth or to a different mechanism of growth.

In the varieties of grapes studied here, only the seeded varieties, Muscat and Emperor, show a correlation between meristematic activity in the seed, level of auxins and the first cycle of berry growth. In Sultanina, the auxin level parallels the rate of cell division in the seed but the rate of increase in berry volume is maintained at a high level 10 days longer. The situation in Seedless Emperor is not as clear because no detailed study of seed development was made but, in any case, auxin content remained low while the berries grew. In Corinth there was a sharp peak in auxin content 11 days after anthesis when there was no seed development at all. It seems more likely that the growth of these seedless grapes is controlled by gibberellin levels rather than auxins or that gibberellins act together with auxins.

The second cycle in berry growth does not coincide with a rise and fall of either auxins or gibberellins. As suggested above, a more acceptable hypothesis to explain this growth cycle is that the movement of sugars into the berries causes an osmotic attraction of water.

Summary

Measurements were made of growth and development of certain parts of the fruit and seed of two seeded and three seedless varieties of *Vitis vinifera* (European grape) from anthesis to maturity. These were compared with changes in the sugar concentration of the juice and the levels of auxins and gibberellins in partially-purified extracts of berries.

The fruit of all varieties showed a double-sigmoid growth curve. In the two seeded varieties, the first growth cycle was paralleled by a rise and fall in meristematic activity in the seeds and in the auxin content of the berries. In the seedless berries, the first growth cycle was greater than would be expected from the berry's auxin content and the seed's meristematic activity. This may be explained by the discovery of gibberellin activity in the young berries of the three seedless varieties. No gibberellin activity was found in seeded berries at any time. These findings, plus the fact that berries of seedless varieties enlarge considerably after treatment with gibberellin, auxin, and by girdling, whereas seeded varieties do not, suggest that gibberellins are important hormones in the fruit of these seedless varieties. Mature stamens of all varieties showed gibberellin activity.

The second growth cycle could not be correlated with morphological, auxin, or gibberellin changes but could be related to the influx of sugars into the berries. It is suggested that sugaring causes this growth cycle by an osmotic attraction of water.

A second rise in auxin level was found to reach a maximum in four varieties at the beginning of sugaring. These events may be related but no explanation can be given for this phenomenon. Trunk girdling of two seedless varieties caused no measurable change in auxin or gibberellin concentration expressed on dry weight, fresh weight, or volume bases, but levels per berry increased because of the large increase in berry volume after girdling.

Acknowledgements

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Literature Cited

1. Bentley, Joyce A. 1958. The naturally-occurring auxins and inhibitors. Ann. Rev. Plant Physiol. 9: 47–80.
2. Crane, J. C., Muriel V. Bradley, and L. C. Luckwill 1959. Auxins in parthenocarpic and non-parthenocarpic figs. Jour. Hort. Sci. 34: 142–153.
3. Crane, J. C. and J. G. Brown 1950. Growth of the fig fruit, *Ficus carica* var. Mission. Proc. Am. Soc. Hort. Sci. 56: 93–97.
4. Crane, J. C., E. D. de Kazos, and J. G. Brown 1956. The effect of 2,4,5-trichlorophenoxyacetic acid on growth, moisture, and sugar content of apricot fruits. Proc. Amer. Soc. Hort. Sci. 68: 105–112.
5. Ferenczy, L. 1957. Examination of ether extractable growth substances in grape and watermelon with paper chromatography. Phyton (Buenos Aires) 9: 47–52.
6. Gustafson, F. G. 1939. The cause of natural parthenocarpy. Amer. Jour. Bot. 26: 135–138.
7. Horsfall, J. G. and A. E. Dimond 1957. Interactions of tissue sugar, growth substances, and disease susceptibility. Z. Pflanzenkrankh. u. Pflanzenschutz. 64: 415–421.
8. Jacob, H. E. 1928. Some responses of the seedless varieties of *Vitis vinifera* to girdling. Proc. Amer. Soc. Hort. Sci. 25: 223–229.
9. Kissler, J. J. 1957. Nitrate fluctuations and petiole sampling technique with grapevines. M.S. Thesis, University of California, Davis.

10. Luckwill, L. C. 1948. The hormone content of the seed in relation to endosperm development and fruit drop in the apple. Jour. Hort. Sci. 24: 32–44.
11. Neely, P. M. and B. O. Phinney 1957. Use of mutant dwarf-1 of maize as a quantitative bioassay for gibberellin activity. Plant Physiol. 32 suppl.: xxxi.
12. Nitsch, J. P. 1953. The physiology of fruit growth. Ann. Rev. Plant Physiol. 4: 199–236.
13. Nitsch, J. P. 1955. Free auxins and free tryptophane in the strawberry. Plant Physiol. 30: 33–39.
14. Nitsch, J. P. and Colette Nitsch 1955. The separation of natural plant growth substances by paper chromatography. Beitr. Biol. Pflanz. 31: 387–408.
15. Nitsch, J. P. and Colette Nitsch 1956. Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight-growth test for auxins. Plant Physiol. 31: 94–111.
16. Nitsch, J. P., Colette Nitsch, Charlotte S. Pratt, and N. J. Shaulis 1957. Auxins in the Concord and Concord Seedless grapes in relation to berry development and drop. Plant Physiol. 32 suppl.: xx.
17. Olmo, H. P. 1937. Pollination and the setting of fruit in the Black Corinth grape. Proc. Amer. Soc. Hort. Sci. 34: 402–404.
18. Olmo, H. P. 1940. Somatic mutation in the vinifera grape. III. The Seedless Emperor. J. Heredity 31: 211–213.
19. Overbeek, J. van 1959. Auxins. Bot. Rev. 25: 269–350.
20. Pearson, Helen M. 1932. Parthenocarpy and seed abortion in *Vitis vinifera*. Proc. Amer. Soc. Hort. Sci. 29: 169–175.
21. Phinney, B. O. and P. M. Neely 1958. Differential biological properties of gibberellin-like factors isolated from beans and peas. Plant Physiol. 33 suppl.: xxxviii.
22. Phinney, B. O., C. A. West, Mary Ritzel, and P. M. Neely 1957. Evidence for gibberellin-like substances from flowering plants. Proc. Nat. Acad. Sci., U. S. 43: 398–404.
23. Shaulis, N. J. 1959. Gibberellin trials for New York grapes. Farm Res. 25: 11.
24. Stout, A. B. 1936. Seedlessness in grapes. N.Y. Agr. Exp. Stn. Tech. Bull. No. 238: 1–68.
25. Thomas, Meirion, S. L. Ranson, and J. A. Richardson 1956. Plant Physiology. (4th Edit.) 692 pp. Philosophical Library, New York
26. Walker, D. R., C. H. Hendershott, and G. W. Snedecor 1958. A statistical evaluation of a growth substance bioassay method using extracts of dormant peach buds. Plant Physiol. 33: 162–166.
27. Weaver, R. J. 1953. Furthur studies on effects of 4-chlorophenoxyacetic acid on development of Thompson Seedless and Black Corinth grapes. Proc. Amer. Soc. Hort. Sci. 61: 135–143.
28. Weaver, R. J. and S. B. McCune 1959. Response of certain varieties of *Vitis vinifera* grapes to gibberellic acid. Hilgardia 28: 297–350.
29. Weaver, R. J. and S. B. McCune 1959. Girdling: its relation to carbohydrate nutrition and development of Thompson seedless, Red Malaga, and Ribier grapes. Hilgardia 28: 421–456.
30. West, C. A. and Kate H. Murashige 1958. The isolation of gibberellin A_1 from beans and the chemical properties of other gibberellin-like factors from beans and peas. Plant Physiol. 33 suppl.: xxxviii.
31. Winkler, A. J. 1930. Berry thinning of grapes. Cal. Agr. Exp. Stn. Bull. No. 492: 1–22.
32. Winkler, A. J. 1953. Producing table grapes of better quality. Blue Anchor. 30: 28–31.
33. Winkler, A. J. and W. O. Williams 1936. Effect of seed development on the growth of grapes. Proc. Amer. Soc. Hort. Sci. 33: 430–434.
34. Wright, S. T. C. 1956. Studies of fruit development in relation to plant hormones. III. Auxins in relation to fruit morphogenesis and fruit drop in the black currant, *Ribes nigrum*. Jour. Hort. Sci. 31: 196–211.

INFLUENCES OF GIBBERELLIC ACID ON METABOLISM OF INDOLEACETIC ACID, ACETATE, AND GLUCOSE IN ROOTS OF HIGHER PLANTS [1, 2, 3]

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The major effects of the gibberellins (GA) on plants have been reviewed recently (13, 14) but no definite linkage with any metabolic pathway has been established. In their study of photosynthesis in gibberellin treated leaves Haber and Tolbert (8) concluded that gibberellic acid neither enhanced the rate of CO_2 fixation per unit of leaf tissue nor altered the general pathways of short time metabolism of the newly fixed $C^{14}O_2$ in the sugars, organic acids, and amino acid products. In long term experiments, great changes in carbohydrate constituents of gibberellin treated plants have been reported (4, 9). Inhibition in the level of indoleacetic acid oxidase by gibberellic acid has been reported by Pilet (12). However, Brian and Hemming (5) did not find a similar effect in their system. Recently, Galston and Warburg (7) suggested a possible action of gibberellin through an auxin-sparing mechanism by way of the formation of IAA oxidase inhibitor. It is the purpose of this paper to describe the results of experiments on the influence of GA on plant metabolism. In the method used, plant tissue was first pre-treated with GA and then incubated with a selected isotopically labeled substrate. Samples of the tissue were then harvested after several hours of incubation. The influence of GA on the metabolism of the material was determined by comparing the extents of the labeled atoms which have been metabolized and incorporated into other compounds between the control and treated tissues.

MATERIALS AND METHODS

Alaska pea (*Pisum sativum* L.) and sweet corn (*Zea mays* L. var. Golden Cross) were soaked in distilled water for 2 hours and were then germinated in the dark between two sheets of moist paper. Uniform seedlings were removed from the paper after two or three days and the roots were immersed in either 0.01 M phosphate buffer (pH 5.2) or buffer + 5 ppm GA. At the end of the 2 hours pre-treatment period, the seedlings were removed from the solution and then rinsed with water. The distal 2 cm of the roots was cut off, blotted dry, and weighed. Equal samples of root tips were placed in 50 ml Erlenmeyer flasks which contained 10 ml of 0.01 M phosphate buffer, pH 5.2, along with C^{14} labeled substrates, and were allowed to incubate at 26 to 28° C. The respiratory $C^{14}O_2$ was collected periodically as described previously (6). At the end of the incubation period, the tissues were removed from the medium, rinsed with water, and homogenized with ethyl alcohol. The radioactivities of the medium, alcohol extract of the tissues, and the residue of the tissue were also determined by the usual method.

The incorporation of C^{14} in each isotopically labeled metabolite was studied by paper chromatographic and radioautographic techniques.

RESULTS AND DISCUSSION

EFFECT OF GA ON METABOLISM OF IAA-1-C^{14} The influence of GA on the metabolism of auxins in higher plants has always been of interest. This problem was approached by using C^{14} labeled indoleacetic acid. Corn root tissue has been chosen for this study because in addition to the decarboxylative oxidation, C–1 carbon of IAA has been found to incorporate into many metabolites. The root tips from control and GA treated seedlings were incubated separately at 26° C in 10 ml 0.01 M phosphate buffer (pH 5.2) containing 2.0×10^{-5} M IAA-1-C^{14}. Twelve 40-watt fluorescent lights placed 3 ft from the flask, providing a light intensity of 800 ft-c, were used for illumination during the incubation period. Respiratory CO_2 was collected periodically and precipitated as $BaCO_3$. After a 6 hr period of incubation, the root tissues were removed, rinsed, and homogenized with 10 ml ethyl alcohol. The alcohol extract was filtered and the residue was washed thoroughly with alcohol. The radioactivities of $BaCO_3$, alcohol extract, and insoluble residue were determined.

The data from two separate experiments are shown in table I. There was no significant difference in the total absorption of IAA by the control and GA treated tissues. However, the ratio (O/A) between the decarboxylative oxidation and the absorption of IAA was slightly higher in GA treated tissues indicating that oxidative degradation of IAA might be affected. The time course radiochemical recovery of respiratory CO_2 revealed that a noticeable increase in oxidation by GA treated tissue occurred only during the first 2 hours. The cumulative recoveries of respiratory CO_2

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[3] Technical Paper No. 1256, Oregon Agricultural Experiment Station.

TABLE I

EFFECT OF GA ON DISTRIBUTION OF RADIOACTIVE CARBON FROM IAA-1-C^{14} BY CORN ROOT TIPS

EXPT	TISSUE WT	TREATMENT	TOTAL ABSORPTION IAA-1-C^{14}	O/A	% RECOVERY OF C^{14} IN VARIOUS FRACTIONS FROM ABSORBED IAA-1-C^{14}		
					RESPIRATORY CO_2	ETHANOL EXTRACT OF TISSUE	RESIDUE OF TISSUE
	mg		*μg*				
1	651	Buffer	18.9	0.50	50.2	44.5	5.3
	687	GA + Buffer	18.6	0.53	52.8	39.0	8.2
2	637	Buffer	20.8	0.49	48.8	42.4	8.7
	659	GA + Buffer	22.2	0.53	52.8	38.7	8.5

from control and GA treated tissues were quite similar (fig 1). The incorporation of isotopic carbon from IAA-1-C^{14} into various metabolites as revealed from paper chromatographic and radioautographic studies is presented in table II. Results indicated that the formation of indoleacetylaspartate was significantly reduced. The free IAA-1-C^{14} concentration as calculated from the experimental data was 15 % higher in GA treated tissue (3.07 μg/1 gm fresh tissue) than in the control tissue (2.69 μg/1 gm fresh tissue). If the concentration of absorbed IAA in tissue could be an indication for the endogenous auxin level, then higher endogenous IAA in GA treated tissue would be expected. This observation provides further evidence in support of auxin-gibberellin interaction. It was not in complete agreement with the report by Pilet (12), however, that the increase of IAA level in GA treated tissue was a result of inhibition of IAA oxidase but rather from a result of the decreasing formation of indoleacetylaspartate. Hayashi and Murakami (9) in bioassays of extractable and diffusible auxins from several different plants, could find no change in auxin levels after GA treatments. Since indoleacetylaspartate is as effective as IAA either in curvature or in elongation tests with oat coleoptiles (1), measurements of extractable and diffusible auxins by a bioassay method would not differentiate this transformation and the concentration of each. No difference was observed in other radioactive metabolites between the control and GA treated tissues.

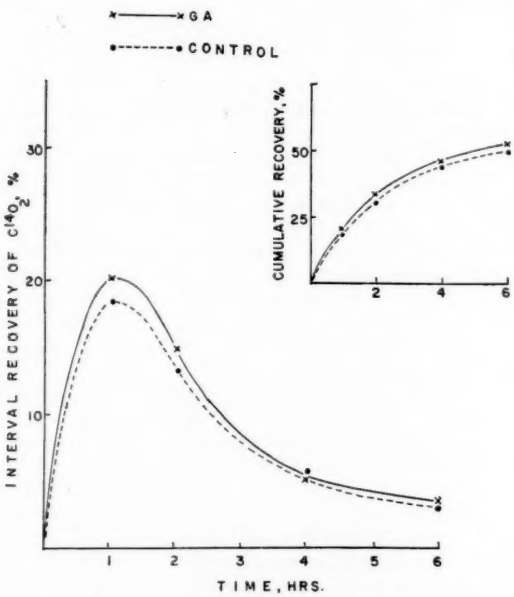

FIG. 1. Time course plot of radiochemical recovery in respiratory CO_2 from indoleacetic acid-1-C^{14} by control and GA treated corn root tissues.

EFFECTS OF GA ON GLUCOSE METABOLISM Several experiments similar to those described in the preceding section were also carried out with pea root sections with either glucose-U-C^{14}, glucose-1-C^{14} or glucose-6-C^{14} as substrate at a concentration of 1 mg glucose per 10 ml medium. The root tissues (1.3–1.4 gm fresh weight) were incubated for 6 hours. At the end of incubation, the reaction was stopped by adding 0.5 ml 6 *N* H_2SO_4. Shaking was continued for another 20 minutes in order to remove completely the respiratory CO_2. Tissues were harvested, rinsed, and ground with ethyl alcohol. Radioactivities of respiratory CO_2, alcohol extract, and alcohol insoluble residue were determined. The alcohol extracts were chromatographed unidimensionally with *n*-butanol-

TABLE II

PAPER CHROMATOGRAPHIC STUDY OF FORMATION OF RADIOACTIVE METABOLITES IN CORN ROOT TISSUES INCUBATED IN IAA-1-C^{14} SOLUTION

	DISTRIBUTION OF RADIOACTIVITY % TOTAL						
	A R_fO	B* 0.03	C 0.05	D 0.10	E 0.14	F** 0.27	G 0.60
Control	26	38	14	T	T	20	T
GA	24	28	15	2	T	26	4

* Spot B is indoleacetyl aspartic acid.
** Spot F is indoleacetic acid.

acetic acid-water solvent (4: 1: 1 v/v). Radioautograms of the resultant chromatograms revealed the presence of three principal components. These components appeared identical with respect to Rf values and relative intensities as between the GA treated and non-GA treated tissues. Table III shows the results on total absorption of substrate, and C^{14} recoveries in respiratory $C^{14}O_2$, alcohol insoluble tissue constituents, and alcohol extract. Five independent experiments were carried out with glucose-U-C^{14} and three experiments each with glucose-1-C^{14} and glucose-6-C^{14}. The results of these experiments were in close quantitative agreement throughout and therefore the average values are presented for comparison. It is apparent from the data of Table III that there is no significant difference between the control and GA treated tissues either on the total absorption of substrate or in the percent distribution of C^{14} in various fractions. It also is apparent that an appreciable portion of the labeled carbon supplied to pea root tissue as glucose becomes incorporated into cell wall constituents (residue insoluble in 80 % ethyl alcohol). Percentagewise, the anabolic functions of C-6 carbon from glucose as measured directly from its incorporation to cellular constituents was higher (47 %) than that of C-1 carbon (34 %). In contrast, the participation of the catabolic functions of carbon 1 of glucose as measured from the radioactivity of respiratory CO_2 revealed that it was 20 % greater than the carbon 6. Approximately 10 % of the radioactivity from the absorbed glucose in root tissue can be extracted by 80 % ethanol from which three prominent components including the unchanged glucose have been revealed by paper chromatography and radioautography. The average value of C_6/C_1 ratio derived from respiratory CO_2 from control pea root tissue is 0.68 ± 0.03 (3 runs) which is in agreement with the value (0.74 ± 0.11) as reported by Humphreys and Dugger (10). Pre-treatment of pea roots with GA did not alter the C_6/C_1 (0.70 ± .05) appreciably, thus indicating that the pathways of glucose catabolism are not affected. When the yields of C^{14} incorporation into cell wall constituents from C–1 and C–6 of glucose were compared, the result again indicated that no appreciable differences were found between the control tissue (1.47) and GA treated ones

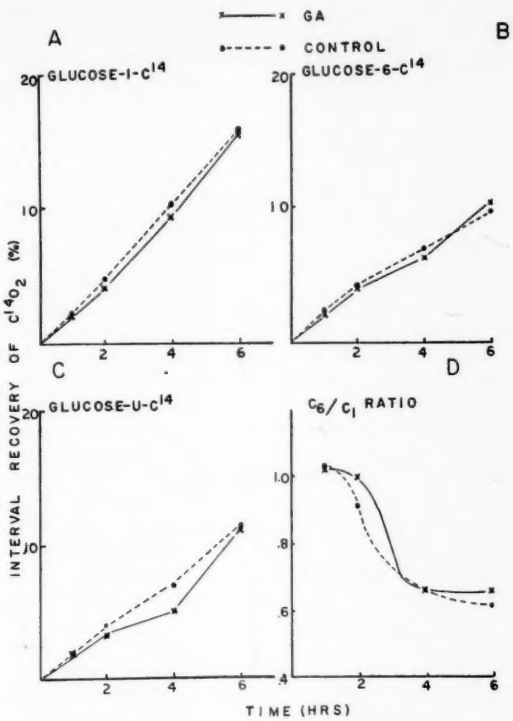

FIG. 2. Time course plots of radiochemical recoveries in respiratory CO_2 from C^{14} labeled glucose by control and GA treated pea root tissues.

(1.31). It is therefore concluded that a short term GA treatment of pea roots will neither alter their pathways of glucose catabolism nor exert any gross effect in the incorporation of carbon atoms of glucose into other major cellular constituents. The time course recovery of radioactivity in respiratory CO_2 from the control and the GA treated pea root tissues metabolizing C^{14} labeled glucose were shown in figure 2 which provided further evidence that no significant effect either on the combustion of C–1 or C–6 of glucose was observed at any given time during the ex-

TABLE III

EFFECT OF GA ON UPTAKE AND UTILIZATION OF GLUCOSE BY PEA ROOT TIPS

C^{14} SUBSTRATE	TREATMENT	NO. RUNS	WEIGHT TISSUE USED	TOTAL ABSORPTION OF GLUCOSE	RECOVERY OF C^{14} IN VARIOUS FRACTIONS FROM ABSORBED GLUCOSE		
					RESPIRATORY CO_2	ALCOHOL EXTRACT OF TISSUE	ALCOHOL INSOLUBLE RESIDUE
			gm	%	%	%	%
Glucose-U-C^{14}	Buffer	5	1.402 ± .043	54.1 ± 4.4	43.4 ± 5.8	10.8 ± 2.7	45.8 ± 2.0
Glucose-U-C^{14}	GA + buffer	5	1.387 ± .037	51.3 ± 2.8	41.8 ± 2.9	13.1 ± 3.8	45.1 ± 2.7
Glucose-1-C^{14}	Buffer	3	1.394 ± .002	66.0 ± 3.6	57.8 ± 2.2	9.4 ± 1.9	32.8 ± 2.0
Glucose-1-C^{14}	GA + buffer	3	1.396 ± .003	63.5 ± 3.3	56.2 ± 2.8	8.5 ± 0.8	35.3 ± 2.4
Glucose-6-C^{14}	Buffer	3	1.396 ± .002	55.2 ± 2.8	39.1 ± 2.4	12.6 ± 2.3	48.3 ± 2.3
Glucose-6-C^{14}	GA + buffer	3	1.395 ± .002	53.5 ± 2.9	39.2 ± 1.3	14.5 ± 1.5	46.3 ± 1.5

perimental period. Furthermore, it was noted that the C_6/C_1 ratios during the first 2 hours of incubation were near unity, indicating that glucose was catabolized in these tissues mainly via the glycolytic path (1st hr 1.07, 2nd hr 0.95). However, the C_6/C_1 ratios which calculated from the yields of CO_2 between the third and the sixth hours showed a decrease from 0.68 at 4th hour to 0.63 at 6th hour, suggesting that a shift of the catabolic pathways had taken place, presumably an increase of participation of pentose cycle route.

Effects of GA on Acetate Metabolism Acetate is metabolized by plant tissues via several pathways (3), the carboxyl carbon of acetate appeared rapidly in the Krebs cycle acids (11), and Krebs cycle related amino acids (2). It is also incorporated into the lipides of the *Avena coleoptile* (3). To investigate the influence of GA on acetate metabolism, the pea root tissues (control and GA treated) were incubated separately in phosphate buffer, pH 5.2, containing 0.5 mg sodium acetate (acetate-1-C^{14} or acetate-2-C^{14}) for 4 hours at 26° C. The respiratory $C^{14}O_2$ was trapped in 0.5 M NaOH solution and determined hourly. At the end of a 4 hour incubation period, the tissues were harvested, rinsed, and ground with 80 % alkali ethanol to assure that no acetate in the tissue would be lost. The radioactivity of $BaCO_3$, alcohol extract and alcohol insoluble residue was determined in the usual manner. After the initial counting, the planchet containing alcohol extract was acidified with a small amount of acetic acid and allowed to dry completely in order to remove the radioactive acetate. The planchet was then again counted. The difference in radioactivity was recorded as free acetate. The alcohol extract was subjected to ascending paper chromatographic separation. The *n*-propanol-ethanol-ammonium carbonate buffer solvent system was employed (15). The radioautographs prepared from the paper chromatograms revealed the detail incorporations of isotopic carbon from either acetate-1-C^{14} or acetate-2-C^{14}, but in no case were there any gross differences found between GA treated and control tissues. Therefore, it is evident that GA does not exert any important effect on the conversion of acetate carbon into any of the major products of acetate metabolism. Six separate experiments were carried out with acetate-1-C^{14} and four experiments with acetate-2-C^{14}. The average values of total absorption, respiratory CO_2, alcohol insoluble residue, and alcohol extract are shown in table IV. The total uptake of acetate by GA treated tissue was consistently lower than that of the control, although the differences were small. The oxidation of either C_1 or C_2 carbons of acetate was slightly increased in GA treated tissue. The catabolic functions of C–1 carbon of acetate were much greater than that of C–2 as indicated from the percent radiochemical recovery in respiratory CO_2. Again, it was noted that considerably more activity has been incorporated into cellular constituents from C–2 carbon of acetate, indicating that the participation of C–2 of acetate in direct biosynthetic functions was greater than that of C–1. GA treatment of pea root tissues revealed a slight increase in the utilization of acetate. Since results from several runs did not show any change, this small increase may not be considered significant.

Summary

The effects of gibberellic acid on the metabolism of indoleacetic acid, glucose and acetate in roots of higher plants have been studied by using C^{14} labeled compounds.

GA, at the concentration of 5 ppm increased slightly $C^{14}O_2$ production from administered IAA-1-C^{14} in corn root tissues. The formation of indoleacetylaspartate was greatly inhibited and consequently the free IAA level in GA treated tissues was increased by 15 % over the control tissues.

The catabolic pathways of glucose and acetate in pea root tissue as examined by the tracer method were not found to be affected to any significant extent by short-term GA treatment. However, GA treatment slightly increased the catabolic oxidation of C–1 and C–2 carbons of acetate.

Table IV

Effect of GA on Uptake of Utilization of Acetate by Pea Root Tips

C^{14} Substrate	Treatment	No. runs	Weight tissue used	Total absorption	Recovery of C^{14} in various fractions from absorbed acetate-C^{14}			
					Respiratory CO_2	Alcohol extract of tissue including acetate	Free acetate in tissue	Alcohol insoluble residue
			mg	%	%	%	%	%
*Acetate-1-C^{14}	Buffer	4	992 ± 6	50.4 ± 4.8	55.0 ± 0.9	30.8 ± 1.2	11.4 ± 0.6	14.2 ± 1.0
*Acetate-1-C^{14}	GA + buffer	4	974 ± 22	45.6 ± 2.0	62.6 ± 3.3	24.2 ± 2.2	9.7 ± 0.5	13.2 ± 0.7
Acetate-1-C^{14}	Buffer	2	1,117	75.7	47.5 ± 0.5	32.3 ± 1.5	4.8	20.2 ± 1.9
Acetate-1-C^{14}	GA + buffer	2	1,066	72.4	49.6 ± 0.4	27.8 ± 0.5	2.8	22.7 ± 1.0
Acetate-2-C^{14}	Buffer	4	1,105 ± 8	70.7 ± 4.3	27.2 ± 7.2	42.9 ± 8.2	2.9	29.8 ± 2.0
Acetate-2-C^{14}	GA + buffer	4	1,103 ± 13	63.9 ± 5.1	29.8 ± 5.1	35.8 ± 6.4	3.3	34.0 ± 2.6

* Reaction was stopped by adding H_2SO_4 after 4 hrs.

Chromatography and radioautography of the alcohol soluble constituents of pea root tissues which had been incubated with either acetate-1-C^{14}, acetate-2-C^{14}, or glucose-U-C^{14} failed to reveal any major effect of short-term GA treatment although the isotopic carbon of all three substances was rapidly incorporated into a variety of compounds in the plant.

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Literature Cited

1. Andreae, W. A. and N. E. Good 1955. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380–382.
2. Arreguin, B., J. Bonner, and B. J. Wood 1951. Studies on the metabolism of rubber formation in the guayule. III. Experiments with isotopic carbon. Arch. Biochem. Biophys. 31: 234–247.
3. Boroughs, H. and J. Bonner 1953. Effect of indoleacetic acid on metabolic pathways. Arch. Biochem. Biophys. 46: 279–290.
4. Brian, P. W., G. W. Elson, H. G. Hemming, and M. Radley 1954. The plant-growth-promoting properties of gibberellic acid, a metabolic product of the fungus *Gibberella funjikuroi*. Jour. Sci. Food Agr. 5: 602–612.
5. Brian, P. W. and H. G. Hemming 1958. Complementary action of gibberellic acid and auxins in pea internode extension. Ann. Bot. 22: 1–17.
6. Fang, S. C., Patricia Theisen, and J. S. Butts 1959. Metabolic studies of applied indoleacetic acid-1-C^{14} in plant tissues as affected by light and 2,4-D treatment. Plant Physiol. 34: 26–32.
7. Galston, A. W. and H. Warburg 1959. An analysis of auxin-gibberellin interaction in pea stem tissue. Plant Physiol. 34: 16–22.
8. Haber, A. H. and N. E. Tolbert 1957. Photosynthesis in gibberellin-treated leaves. Plant Physiol. 32: 152–153.
9. Hayashi, T. and Y. Murakami 1958. Studies on the physiological action of gibberellins. Bull. Nat. Inst. Agr. Sci. (Japan) D. No. 7: 159–197.
10. Humphreys, T. E. and W. M. Dugger, Jr. 1957. The effect of 2,4-dichlorophenoxyacetic acid on pathways of glucose catabolism in higher plants. Plant Physiol. 32: 136–150.
11. Krotkov, G. and H. J. Barker 1958. Utilization of acetate by tobacco leaves, as determined with C^{14}. Amer. Jour. Bot. 35: 12–15.
12. Pilet, P. E. 1957. Action des gibberelins sur l'activité auxin-oxydasique de tissue cultivé in vitro. Compt. rend. 245: 1327–1328.
13. Stowe, B. B. and T. Yamaki 1957. The history and physiological action of the gibberellins. Ann. Rev. Plant Physiology 8: 181–216.
14. Stowe, B. B. and T. Yamaki 1959. Gibberellins: Stimulants of plant growth. Science 129: 807–816.
15. Van Duuren, A. J. 1953. Determination of organic acids in plant material by paper chromatography. Rec. trav. chim. 72: 889–92.

SEPARATION OF PLANT GROWTH REGULATING SUBSTANCES ON SILICA GEL COLUMNS [1, 2]

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Silica gel columns have been successfully employed to separate a number of synthetic indole derivatives[3] and naturally occurring plant growth regulators. The method is rapid and simple, and appears to be superior to paper chromatography in many respects for this type of work.

MATERIALS AND METHODS

The preparation of the silica gel is similar to the method of Bulen, et al (2) for organic acid chromatography. Silicic acid (Mallinckrodt's 100 mesh analytical reagent) is prepared, generally in one pound batches, by repeatedly washing it in distilled water, and decanting the fine particles that do not settle out of suspension after an arbitrarily chosen period of 15 minutes. After the silicic acid (silica gel) is free of very fine particles that greatly impede solvent flow through the column, it is dried to constant weight at 100° C.

A glass column 1.4 cm inside diameter and 30 cm long, with a 250 ml solvent reservoir on one end and stopcock on the other, is used to support the silica gel bed. A small plug of fine glass wool at the bottom of the column prevents the silica gel from entering the stopcock.

An 8.0 g sample of the dried silica gel is hydrated with 5.0 ml of 0.5 M formic acid. This hydrated sample is suspended in a small amount of the 0.2 % butyl alcohol eluting solvent (petroleum ether: *n*-butyl alcohol saturated with 0.5 M formic acid–99.8: 0.2), poured into the column, and packed with air pressure applied to the top of the column. This results in a column of silica gel approximately 13 cm long.

The sample to be analyzed is placed on top of the column in one of three ways: I. The finely ground tissue sample is mixed with 0.5 g of silica gel which has previously been hydrated with 0.4 ml of 0.5 M formic acid, and transferred directly to the top of the column. This is the method generally used. If the tissue sample has previously been extracted, the liquid extract may be reduced to a small volume and applied to the top of the column, either by II. pipetting it directly on to the column, or III. first mixing it with a small sample of silica gel which has previously been hydrated with formic acid. Method No. II is acceptable if the extracting solvent does not undesirably alter the elution pattern of the regular eluting solvents. If it does, it may be necessary to mix the extract with a small sample of silica gel which has previously been hydrated with formic acid, then carefully evaporate the extracting solvent from the sample with as little loss of the hydrating liquid as possible. As explained in a later section, dehydrated silica gel may account for a substantial loss of certain substances.

The prepared column is placed on a fraction collector adjusted to collect 10 ml fractions. One hundred fifty ml of the 0.2 % butyl alcohol eluting solvent is added to the solvent reservoir, and the air pressure on top of the column regulated to an elution rate of 2 to 3 ml per minute. The first eluting solvent is followed by similar solvents made increasingly polar with butyl alcohol. For routine survey work a suggested solvent schedule is shown in table I. After it has been determined which of these solvents are important for any particular material the others may be eliminated from the solvent schedule, thus shortening the chromatographic process.

The eluate is evaporated at a reduced pressure from the test tubes in which the various fractions have been collected. The residues in the test tubes are bioassayed for plant growth regulating substances using the Avena first-internode test as introduced by Nitsch and Nitsch (8), or by some other satisfactory testing procedure. For the colorimetric detection of indole derivatives the Gordon-Weber modification of the Salkowski reagent (4), and Ehrlich's reagent (3) are employed. The Salkowski reagent is made by dissolving 5.40 g of ferric chloride in 21 of 35% perchloric acid. Two milliliters of this solution are used for each milliliter of aqueous solution containing the indole derivative. Ehrlich's reagent consists of 10.0 g of *p*-dimethylaminobenzaldehyde dissolved in 100 ml of concentrated hydrochloric acid, sp. gr. 1.188. Two milliliters of this reagent are used for each milliliter of aqueous solution containing the indole derivative, except in the case of indole where the reverse ratio is better.

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[1] Received revised manuscript July 27, 1959.

[2] Approved by the director of the N. Y. State Agricultural Experiment Station for publication as Journal Paper No. 1152, March 16, 1959.

[3] The following abbreviations are used in the figures: IAE, ethyl indoleacetate; IAN, indoleacetonitrile; IBA, indolebutyric acid; IPA, indolepropionic acid; IAA, indoleacetic acid; IAM, indoleacetamide.

Results

Most of the early work involved in the development of this method employed water to hydrate the silica gel. The method was eventually modified to include 0.5 M formic acid as the hydrating agent in place of the water. Some of the data to be presented are from the early experiments, while tables I and II, and figures 1 and 4 include data from experiments in which formic acid was employed.

It was discovered early in this work that eluting solvents with a nonpolar base, such as petroleum ether, are well suited to chromatography of indole derivatives on silica gel columns. By adding varying amounts of a more polar solvent, such as *n*-butyl alcohol, it is possible to make solvent mixtures which will separate a considerable number of indole derivatives of widely varying characteristics. These solvents must be saturated with an aqueous solution to prevent them from removing the stationary aqueous phase from the column itself.

Care must be exercised that the column is not exposed to temperatures of greater than 30° C. when solvents containing petroleum ether are used, otherwise the boiling point will be exceeded, with resultant cracking of the column. Limited experiments with *n*-hexane indicate that it can be substituted for the petroleum ether in the various solvents, the substitution possibly requiring minor changes in the *n*-butyl alcohol concentration in the 0.2 % butyl alcohol solvent to effect better separation of ethyl indoleacetate and indoleacetonitrile. Hexane, B.P. 68.7° C., overcomes the column-cracking problem associated with elevated laboratory temperatures, yet is relatively easy to evaporate.

A solvent schedule was derived, primarily by empirical methods, which would permit the separation of several indole derivatives. In table I are listed these solvents, the suggested volumes to use, and some indole derivatives eluted by each. Figure 1 shows the elution pattern of these indole compounds when such a solvent schedule is used. The 0.2 % solvent separates and elutes certain neutral indole derivatives such as ethyl indoleacetate and indoleacetonitrile. Indole and skatole are very readily eluted, being nearly on the solvent front. They are not separated from each other by this solvent, however, nor are they separated by completely eliminating the *n*-butyl alcohol from the solvent. The 3 % solvent elutes such acidic substances as indolebutyric acid, indolepropionic acid, and indoleacetic acid. Indolebutyric acid and indolepropionic acid are not separated, but can be eluted separately by using a 2 % solvent. However, the 2 % solvent does not elute indoleacetic acid in as sharp a zone as the 3 % solvent. The 10 % solvent elutes indoleacetamide, and the 75 % solvent trypotophan and tryptamine. The last two substances are

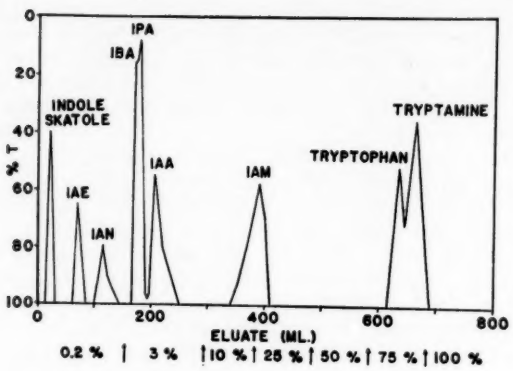
Table I

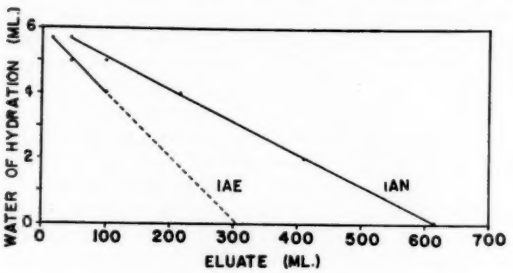
Solvent Schedule for Chromatography of Indole Derivatives on Silica Gel Column

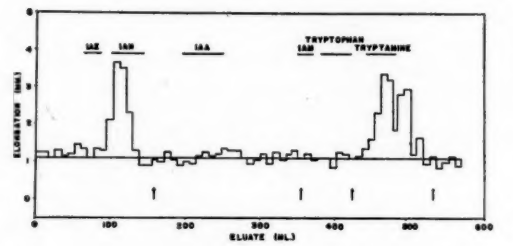
Solvent	Composition	Amount to use (ml)	Indole derivatives eluted
0.2 %	Petroleum ether*: *n*-butyl Alcohol saturated with 0.5 M Formic acid—99.8: 0.2	150	Indole Skatole Ethyl indoleacetate Indoleacetonitrile
3 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—97: 3	150	Indolebutyric acid Indoleopropionic acid Indoleacetic acid
10 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—90: 10	100	Indoleacetamide
25 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—75: 25	100	...
50 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—50: 50	100	...
75 %	Petroleum ether: *n*-butyl Alcohol saturated with 0.5 M Formic acid—25: 75	100	Tryptophan Tryptamine
100 %	*n*-Butyl alcohol saturated with 0.5 M formic acid	100	...

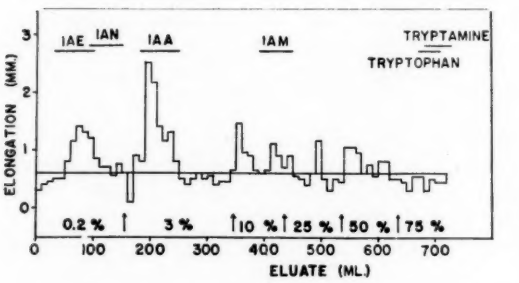
* Mallinckrodt's A.R. petroleum ether, B.P. 30 to 60° C, was used throughout this work without further purification.

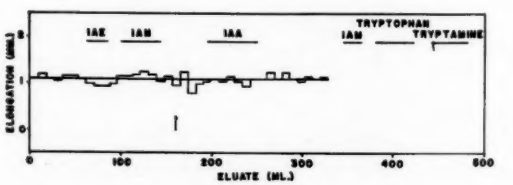
Excess water which separates from solution when the *n*-butyl alcohol and petroleum ether are mixed is removed by centrifugation.











not completely separated with this solvent when an acid-hydrated column is employed, but are well separated by a water-hydrated column. The elution pattern for several indole derivatives on a water-hydrated column may be seen in figure 3. Of the indole derivatives chromatographed the position of only tryptamine is changed by the acid hydration.

First attempts to obtain good recovery of indoleacetic acid after chromatography were not successful. The method used was to pipette 0.5×10^{-6} moles of indoleacetic acid, dissolved in 0.1 ml of absolute ethyl alcohol, into 0.5 g of dry silica gel. This sample was then placed in a vacuum dessicator and held at a pressure of approximately 3 mm mercury for about 30 minutes to remove any traces of alcohol. The dried sample of silica gel plus indoleacetic acid was then hydrated with 0.3 ml of water and placed on top of a silica gel column for chromatography. Under these conditions the recovery rate was never appreciably better than two-thirds. This difficulty was finally overcome by hydrating the 0.5 g sample of silica gel with water or formic acid before adding the indoleacetic acid. Apparently the dehydrated, partially-activated silica gel either adsorbs some of the indole-

FIG. 1 (*top*). Elution pattern of indole derivatives chromatographed on a silica gel column. 0.5×10^{-6} moles of each substance was chromatographed using the following solvent schedule: 0.2 %-150 ml; 3 %-150 ml; 10 %-100 ml; 25 %-100 ml; 50 %-100 ml; 75 %-100 ml; 100 %-100 ml. The Salkowski reagent was used to detect IAE, IAN, IAA, and IAM. Other compounds were detected with Ehrlich's reagent. Silica gel hydrated with 0.5 M formic acid.

FIG. 2 (*second from top*). Volume of 0.2 % solvent required to elute first fraction of IAE and IAN from a silica gel column hydrated with different amounts of water.

FIG. 3 (*third from top*). Silica gel column chromatography of growth substances in cabbage. Solvent schedule: 0.2 %-150 ml; 3%-200 ml; 75 %-75 ml; 100 %-100 ml; water-50 ml. Arrows indicate where solvent changes occurred. Silica gel hydrated with water. Horizontal line running length of histogram indicates growth made by control Avena first-internode sections.

FIG. 4 (*fourth from top*). Silica gel column chromatography of growth substances in peach ovules. Solvent schedule: 0.2%-150 ml; 3 %-200 ml; 10 %-100 ml; 25 %-100 ml; 50 %-100 ml; 75 %-75 ml. Silica gel hydrated with 0.5 M formic acid. Horizontal line running the length of the histogram represents growth made by control Avena first-internode sections.

FIG. 5 (*bottom*). Silica gel column chromatography of a methyl alcohol extract of peach ovules which had previously been extracted on the column by regular eluting solvents. Solvents used for second chromatogram: 0.2 %-150 ml; 3 %-200 ml. Silica gel hydrated with water. Arrow indicates where solvent change occurred. Horizontal line running length of histogram represents growth made by control Avena first-internode sections.

acetic acid so tightly that it cannot be eluted, or inactivates or destroys a part of the indoleacetic acid in some manner. The improved technique permits the recovery of about 90 % of the indoleacetic acid, ethyl indoleacetate, indoleacetonitrile, and indoleacetamide. These results are summarized in table II. The somewhat variable recovery data concerning ethyl indoleacetate is believed due to the fairly rapid fading of the colored compound which develops when the Salkowski reagent is used for detection of this substance.

The separation of certain indole derivatives is affected by the degree of hydration of the silica gel in the column. From figure 2 it is evident that by decreasing the water of hydration greater separation of ethyl indoleacetate and indoleacetonitrile may be obtained. As the amount of hydrating water is decreased the elution fronts for the two compounds become less sharp. That the degree of hydration can be used to alter the elution volumes of indole derivatives which are eluted by solvents other than the 0.2 % solvent has not been investigated, but the method would appear to have its limitations. The solvents containing greater quantities of butyl alcohol also contain larger amounts of acidified water. The silica gel removes the hydrating liquid from the solvent until equilibrium is reached. Therefore, while the degree of hydration of the silica gel remains essentially constant when the 0.2 % solvent, which contains only a trace of water, is used, the water content of the silica gel rapidly tends towards a saturation level when the more polar solvents are employed.

TABLE II

RECOVERY OF SOME INDOLE DERIVATIVES AFTER SILICA GEL COLUMN CHROMATOGRAPHY*

COMPOUND	% RECOVERY
Silica gel hydrated with water after adding indole derivative	
Indoleacetic acid	52.2
" "	66.3
" "	72.8
Silica gel hydrated with water before adding indole derivatives	
Indoleacetic acid	90.2
" "	94.2
Ethyl indoleacetate	101.5
Indoleacetonitrile	91.0
"	88.3
Indoleacetamide	96.8
Silica gel hydrated with 0.5 M formic acid before adding indole derivatives	
Indoleacetic acid	80.7
" "	91.4
" "	92.6
" "	92.1
" "	95.5
" "	96.5
" "	80.6
Ethyl indoleacetate	102.8
" "	126.9
" "	81.8
" "	97.0
Indoleacetonitrile	80.8
"	91.6
"	86.0

* The Salkowski reagent was used to detect the indole compounds.

TABLE III

RECOVERY OF SOME INDOLE DERIVATIVES AFTER SUBJECTING THEM TO MERCURY PRESSURE 3–5 MM FOR 24 HOURS *

COMPOUND	% RECOVERY
Indole	0
Ethyl indoleacetate	95.9
Indoleacetonitrile	92.9
Indoleacetic acid	98.5

* Indole—one test. Ethyl indoleacetate and indoleacetonitrile—mean of four tests. Indoleacetic acid—mean of three tests.

Ehrlich's reagent was used for indole determination. Ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid were detected with the Salkowski reagent.

Evaporating the solvents from the test tubes prior to bioassay is easily accomplished for tubes containing only trace amounts of butyl alcohol by use of a water aspirator connected to a vacuum dessicator in which the tubes have been placed. Of the several methods investigated for the evaporation of appreciable quantities of butyl alcohol, two have proven satisfactory in routine work. Both require a mechanical high-vacuum pump and dry ice trap. The first one employs a rotating vacuum-type evaporator, thus requiring that individual fractions be transferred to the rotating flask, and the residues transferred back to the test tubes with a lower boiling solvent such as methyl alcohol. A more satisfactory method consists of using a vibrating vacuum evaporator (Evapo-Mix, Laboratory Glass and Instrument Corp., N. Y.) designed to evaporate the solvents from ten test tubes simultaneously. This last method is rapid, and eliminates any transferring of residues.

The effect of reduced pressure over an extended period on recovery of certain indole derivatives is summarized in table III. Indole has a rather high vapor pressure, it being impossible to detect any at all after subjecting 1×10^{-6} moles of this substance to a pressure of 3 to 5 mm mercury for 1 hour. It was found that 1×10^{-6} moles of indole had completely disappeared from unstoppered test tubes at atmospheric pressure after 5 days in the dark at $-26°$ C. Experiments with ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid indicated there was little, and perhaps no, significant loss of these materials after 24 hours at a pressure of 3 to 5 mm mercury.

It has occasionally been advantageous to hasten solvent evaporation by use of an infra-red lamp, but caution must be exercised since it has been shown that this method of applying heat may lead to the disappearance, presumably through evaporation, of some of the more volatile substances such as the ethyl ester and nitrile of indoleacetic acid. Indoleacetic acid, itself, does not seem to be affected.

That the silica gel column method is applicable to naturally occurring growth substances in plant material is demonstrated by experiments with cabbage heads and immature peach seeds. Part of a freshly chopped cabbage head was extracted with several portions of ice cold methyl alcohol in a homogenizer, until most of the chlorophyll had been removed. Figure 3 depicts the bioassay pattern of the extract from 21 mg dry weight of the cabbage head after silica gel chromatography, employing Avena first-internode sections (8) to detect the growth substances. Indoleacetonitrile was clearly evident, as were at least two other growth substances eluted in the 100 % solvent. Possibly one of these was the same as referred to by Bentley, et al (1) and Housley and Bentley (5) as a precursor of indoleacetonitrile in cabbage. The precursor referred to by these workers was water soluble, but not ether soluble, and therefore polar in nature. Such a substance might be expected to be eluted by the 100 % solvent. There was no trace of indoleacetic acid; this was further confirmed by analyzing a second cabbage head, using the extract from as much as 2 g fresh weight. These data concerning the absence of indoleacetic acid are in agreement with those of some investigators (5), and in disagreement with the findings of others (6, 7). Possibly varietal or environmental differences exist among cabbages which explain these results. At least two other possibilities need to be considered in explaining its absence. This experiment was performed before formic acid had been adopted as the hydrating agent for the silica gel. Is it possible that indoleacetic acid was present as a salt and was therefore not eluted from the column, or was never extracted by the methyl alcohol in the first place? Secondly, there is a possibility that an inhibitor was present in the test tubes containing the indoleacetic acid, thus masking its activity. This seems unlikely, however, since cabbage extracts of several concentrations were chromatographed; at no time was there any indication of an inhibitor or an auxin in those test tubes in which indoleacetic acid is normally eluted. To postulate that an inhibitor was responsible for the failure to find indoleacetic acid, then, would require an inhibitor whose dosage-response curve in the bioassay was equal to (but opposite in effect) to that of indoleacetic acid.

The growth promoting substances in immature peach seeds, figure 4, showed an entirely different pattern from that of cabbage growth promoters when the same bioassay was employed. Twenty-five milligrams of ground lyophillized peach ovules, of variety Halehaven, representing 0.87 ovules, collected 50 days after bloom shortly after the endosperm had changed from the free-nuclear to the cellular condition, were subjected to column chromatography. At least six growth promoting substances appear to have been eluted. Three of these were eluted in positions similar to ethyl indoleacetate, indoleacetic acid, and indoleacetamide, but their identities have not been established with certainty. Examination of several similar tests of immature peach seeds collected at different stages of development confirm the presence of these substances in certain other samples as well. Their concentrations appear to fluctuate markedly, being correlated, at least in some instances, with the stage of development of the seed.

It may be questioned as to whether extraction is complete when a sample of powdered plant material is placed on top of the silica gel column and extracted during the chromatographic process. To test this a 100 mg sample of peach ovules was first extracted on the column in the usual manner. Previously a similar sample had been shown to have a growth promoter pattern corresponding with the sample described in figure 4 as regards the substances in the ethyl indoleacetate and indoleacetic acid positions. The extracted plant sample was then recovered from the column and ground in a mortar with methyl alcohol, a standard method of extracting auxins for paper chromatography. This methyl alcohol extract was reduced to a small volume and transferred to the top of a silica gel column and chromatographed using the 0.2 % and 3 % solvents only. As figure 5 shows, there were no traces of growth promoting substances. The first extraction apparently completely eluted the growth promoting substances. There are, however, some indications that the column extraction method may not be satisfactory for all plant tissues. Inconclusive, but suggestive evidence from experiments with powdered apple leaves containing rather large amounts of inhibitors indicates that complete extraction may not be obtained with this method.

Discussion

The foregoing results show that certain synthetic indole derivatives (neutral, acidic, and basic) and some naturally occurring growth substances, may be separated on silica gel columns. The method is relatively rapid, and, so far as the author is aware, permits better separation of a number of indole compounds of rather different chemical characteristics than any single-dimension paper chromatographic process now in use. Using the technique described a sample may be extracted and fractionated into neutral, acidic, and basic indole derivatives, and the solvents evaporated from the test tubes preparatory to bioassay, within 10 to 12 hours, under rather mild conditions. Since little or no preliminary purification of the extract is needed before chromatography, the method minimizes transfer of the extract or eluate from one container to another, thus reducing losses and saving time.

The column method can be used with small samples. With the column size described here, cabbage samples of 21 mg dry weight, and peach ovule samples of 25 mg dry weight have been used in conjunction with the Avena first-internode bioassay (8), although less material could have been used satisfactorily. Larger samples can be used but may present certain problems such as overlapping of closely-running substances, and supra-optimal concentrations of biological materials. For much of the work reported

here, either 0.5×10^{-6} or 1×10^{-6} moles of synthetic indole derivatives were used for colorimetric determination, although 1×10^{-7} moles or less is adequate, at least for some substances. When bioassay with the Avena first-internode test was used, 1×10^{-9} moles of ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid were satisfactory.

When a particular solvent does not give satisfactory resolution, better results may often be obtained by changing the amount of *n*-butyl alcohol in the solvent. Changing the fraction size collected is generally less helpful. Employing a column of different length, or changing the degree of hydration of the silica gel may be a solution to some problems.

Experience with this method to date indicates that, contrasted with paper chromatography, the chief disadvantages are minor. Controls cannot be run simultaneously with the chromatography of the extract, such as can be done with paper chromatography, making it necessary to rely on the chromatographic pattern of control substances from another experiment as a guide to where various substances will be eluted. Internal standards that may exist or be placed in a sample, such as pigments, however, aid in relating the behavior of the chromatographic process from sample to sample. Generally the threshold volumes of elution for a particular substance does not vary more than a few milliliters between tests. The fact that reduced pressure is employed to hasten evaporation of solvents from the test tubes causes some loss of volatile constituents, but such losses have been shown to be negligible for ethyl indoleacetate, indoleacetonitrile, and indoleacetic acid.

Summary

Silica gel column chromatography has been successfully employed to separate a number of indole derivatives and naturally occurring plant growth regulating compounds. By placing the tissue sample to be analyzed on top of the column, it is possible to extract and separate several neutral, acidic, and basic indole derivatives, and naturally occurring growth regulating substances, in one operation by a suitable choice of solvents. A bioassay is carried out in the test tubes used to collect the various fractions, thus eliminating the transfer of plant extract from one container to another. The method is simpler and faster than a comparable fractionation employing paper chromatography. It is quantitative, at least for certain substances.

Acknowledgements

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Literature Cited

1. Bentley, J. A., S. Housley, and G. Britton 1956. Hormones and hormone precursors in leaves, roots, and seeds. In: The Chemistry and Mode of Action of Plant Growth Substances, R. L. Wain and F. Wightman, eds. Butterworths Scientific Publications, London.
2. Bulen, W. A., J. E. Varner, and R. C. Burrell 1952. Separation of organic acids from plant tissues. Anal. Chem. 24: 187–190.
3. Ehrlich, F. and K. A. Jacobsen 1911. Conversion of amino acids into hydroxy acids by means of microorganisms. Ber. deut. Chem. Ges. 44: 888–897.
4. Gordon, S. A. and R. P. Weber 1951 Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192–195.
5. Housley, S. and J. A. Bentley 1956. Studies in plant growth hormones. IV. Chromatography of hormones and hormone precursors in cabbage. Jour. Exp. Bot. 7: 219–238.
6. Kefford, N. P. 1955. The growth substances separated from plant extracts by chromatography. I. Jour. Exp. Bot. 6: 129–151.
7. Linser, H., H. Mayr, and F. Maschek 1954. Paper chromatography of indole derivatives found in species of Brassica that can cause cell elongation. Planta 44: 103–120.
8. Nitsch, J. P. and Colette Nitsch 1956. Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight-growth test for auxins. Plant Physiol. 31: 94–111.

GROWTH PROMOTION IN PEA STEM SECTIONS. I. STIMULATION OF AUXIN AND GIBBERELLIN ACTION BY ALKYL LIPIDS.[1, 2]

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A section of pea stem after removal from the plant elongates markedly less than the same zone when it is left on the intact plant. No growth factor or combination of known factors restores more than half of this missing growth. This is particularly evident in studies with the gibberellins. The intact pea plants respond to gibberellin with large growth increments whereas sections cut from replicate plants are only slightly stimulated (*6*,11,*22*).

Our previous paper (*20*) showed that several types of fatty acid esters can partly restore the missing growth of the excised sections. The increased growth was found to be due to a stimulation of auxin and gibberellin action by the fatty acid esters, since the latter substances were inactive by themselves. These unexpected findings indicate that lipid substances have a hitherto unappreciated action in plant growth. Their role in normal plant growth regulation may be exerted through a synergistic effect on hormones rather than as hormones in themselves. The present study has been directed at defining the chemical structure required for this synergism.

MATERIALS AND METHODS

Stem sections of *Pisum sativum* L. were used. These were cut 10 mm long from directly below the apex of the third internode at the time the apical bud and leaves were perpendicular to the stem (about seven days of age). The technique has been described elsewhere (*8*, *20*). The standard variety Alaska was employed for comparison with dwarf peas. A survey of dwarf pea varieties revealed that Laxton's Progress was the most suitable for use under our conditions. Some lots of this variety have not been fungus-free. Therefore Phygon (active ingredient, 2,3-dichloronaphthoquinone) has been satisfactorily used as a fungicide during the initial soaking period. Control experiments have shown that the use of Phygon does not affect the growth response of the sections, and Alaska peas which have never needed Phygon treatment respond as well to these fatty growth factors.

All plants were exposed to continuous weak red light from the 3rd day onward (cf. *20*). Plants so exposed are more uniform and have shorter internodes than when grown in the dark. The response to lipids is not markedly influenced by the amount of red light received, either before or during the test period. Ten sections were placed in Petri dishes containing 20 ml of solution and were kept on a slow speed rotary shaker for 24 hours in the dark. Measurements of the length of the sections were then made.

A major technical difficulty in this study has been the need to apply lipid substances to the plant sections via an aqueous emulsion. Simple lipid dispersions result in unstable emulsions, leading to uncertainty as to the amounts of lipid actually available to the plant material. It was found that the most efficient way to disperse a lipid in water is to dissolve it in a relatively small volume of any fat solvent which is also highly soluble in water, and to inject this solution into the aqueous medium. The following solvents have been found suitable: acetonitrile, acetone, dimethylformamide, dimethyl sulfoxide, and tetrahydrofuran. Dimethylformamide and dimethyl sulfoxide, although they have no effect on pea section growth up to 0.1 M, cannot easily be removed from the system. Acetone, tetrahydrofuran, and acetonitrile, having high vapor pressures, can be partly removed from the aqueous medium by simple aeration. Tests of these volatile solvents revealed that acetone frequently and erratically had an inhibitory effect on pea sections. Tetrahydrofuran, while it was the best solvent for many lipid compounds, inhibited the growth of pea sections at initial concentrations above 0.01 M and had to be frequently repurified. Acetonitrile was the most satisfactory solvent and was usable even at initial concentrations of 0.3 M (*20*). This concentration, however, must have been substantially reduced by volatilization during the 24 hour rotation of the solutions on the shaker. As normally employed, the lipid stock emulsions were aerated for 5 to 15 minutes prior to use, so that the actual concentrations must have been even lower. Wächter (*27*) and Denny (*9*) have reported that acetonitrile causes leaf epinasty, but our acetonitrile (Matheson) has never shown any indication of a growth effect on peas under our conditions. It is unlikely that ambient vapor pressures in our experiments ever approached the

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levels that probably prevailed in Wächter's or Denny's bell jar experiments.

A satisfactory lipid emulsion must also remain dispersed. The Tweens could not be used as they proved to be growth stimulants in themselves (20), and since many other alkyl compounds had this property, other commercial emulsion stabilizers were investigated. The best so far encountered are those of the Pluronic and Tetronic types manufactured by the Wyandotte Chemical Corp. These are based on lipid-soluble polyoxypropylene to which water-soluble polyoxyethylene chains are attached. The resulting compounds have molecular weights and properties which depend on the relative ratios of the two components of the molecule. Pluronics L62, L64, P75, P84, and P85 showed toxicity in our system even at 0.01 %, while Pluronics F77, F88, and Tetronic 707 had moderate inhibitory effect at 0.1 % levels. Pluronics F38, P66, F68, and Tetronic 908, however, were only slightly inhibitory at 0.1 % and could be used at somewhat lower concentrations without any apparent effect on the plant material. For uniformity Pluronic F68 was adopted as the standard compound, but the other substances of this last group would probably also have been suitable.

We are indebted to Professor R. P. Geyer of the Harvard School of Public Health for his advice on these matters, and the technique which follows is based on his procedures. A typical emulsion was prepared in this way: 5.4 mg of methyl palmitate and 4 mg of Pluronic F68 were dissolved in 0.25 ml of acetonitrile and injected with a 1 ml syringe fitted with a No. 18 needle into 10 ml of water in which 4 mg of Pluronic F68 had previously been dissolved. The emulsion was then aerated for 5 to 15 minutes to remove much of the acetonitrile. The final stock emulsion is 0.5 mM in methyl palmitate and 0.08 % Pluronic F68.

This emulsion remains stable for days, even in the cold room. For the tests reported here, however, all emulsions were routinely made up immediately prior to the bioassays. For less soluble substances such as the higher alkyl compounds, and in particular their alcohols, warming of both the acetonitrile and the aqueous solution before injection is necessary. Although twice the lipid weight of Pluronic F68 is generally sufficient to ensure a stable emulsion, higher quantities are occasionally required. The amount of acetonitrile can also be increased, or tetrahydrofuran may be used if the lipid material is insufficiently soluble in acetonitrile. In most of the tests the final concentration of Pluronic F68 was set at 0.004 %.

As many of these lipid substances resist normal washing procedures, all glassware was routinely cleaned in a hot acid bath.

Results

Comparison of Section Growth in situ and in vitro: It was first necessary to establish by what margin excised sections fail to match growth of the intact plant. For this purpose 10 mm zones were marked on ten intact pea plants with India ink at the same position from which sections were cut on the replicate plants. The sections were floated in solutions of sucrose, pH 5.5 phosphate buffer, cobalt chloride, indoleacetic acid (IAA), and gibberellic acid (GA_3) at the concentrations which had been found to provide the largest growth increments. Tests have shown these optimum concentrations are not appreciably altered by the presence of the lipid factors. The intact dwarf plants were small enough to be placed flat within 150 mm Petri dishes in 60 ml of solution and were rotated on the shaker in precisely the same manner as the sections. This procedure resulted in a slight geotropic curvature of the third internode as the cotyledons somewhat restricted the plant motion during this period. Apparently this procedure, even though auxin was in contact with the roots, had

Table I

Percentage Growth of 10 mm Zone on Intact Pea Plants Compared to 10 mm Section

Additive (μM)	Alaska (standard)	Laxton's Progress (dwarf-2 expts)	
	Sections		
Controls	52.7 ± 6.7	44.9 ± 5.6	39.9 ± 3.5
GA_3 (0.3)	....	54.6 ± 5.3	39.9 ± 7.9
IAA (1.7)	....	67.5 ± 7.0	65.1 ± 10.5
IAA + GA_3	94.3 ± 16.1	84.3 ± 6.4	70.1 ± 12.0
Ditto + methyl linoleate (68)	....	113.8 ± 7.3	107.4 ± 9.7
(same at 41)	123.3 ± 15.1	....	....
	Intact plants		
Controls	204.9 ± 24.2	132.4 ± 15.3	125.0 ± 16.8
GA_3 (5 % Paste)	262.2 ± 10.8	....	....
IAA + GA_3	....	201.1 ± 14.6	234.9 ± 24.1

All sections and the intact dwarf plants were grown in 1.25 % sucrose + 50 μM $CoCl_2$ + 5 mM KH_2PO_4 (pH 5.5) and additives at concentrations indicated in parentheses. Solutions initially 0.2 M in acetonitrile. Percentage increase in length in 24 hours and standard deviations are indicated.

no deleterious effects since, as in the experiments of Brian and Hemming (5), the growth of dwarf peas was brought up to that of a standard variety. Since the larger intact Alaska pea plants could not be handled conveniently in the same manner a set was left in the growing trays (8) in the darkroom at the same temperature as the sections and treated with 5 % GA_3 in lanolin paste, applied at the base of the third internode. Another set was left untreated as controls. Gibberellic acid applied to the root solution gave only about 20 % of the growth increment caused by the lanolin treatment. This probably was due to the time required for translocation of the gibberellin. The work of many other investigators (cf. 1, 16) has established that externally applied auxin has only a small growth promoting effect on intact pea plants. Our results are listed in table I.

These experiments confirm that the growth response of both Alaska and dwarf pea stem sections to IAA plus GA_3 is similar, but the sections from dwarfs consistently show slightly less growth. The concentration of IAA selected here (1.7 μM) was that which is usually optimal for the sections from dwarfs; in some tests the Alaska sections reached their optimum at a higher (5.7 μM) concentration. Adding methyl linoleate to the bioassay medium causes a statistically significant increase in growth of both section types. None of these treatments, however, brings the growth of either type of section up to the level it would have reached on the intact plant. The discrepancy is shown most strikingly by the data for the GA_3 treated intact plants (their growth is twice that of the maximum elongation of excised sections). Even with the addition of any of the lipid factors studied here the growth of sections remains submaximal, probably requiring another factor or factors normally provided by the rest of the plant. It is possible that the action of GA_3 depends on these additional factors more crucially than does that of IAA, since the sections in this experiment and in others responded more markedly to the auxin (see also table I in both 20 and 21).

Comparing Effectiveness in Growth Promotion of Different Lipids: The growth promotion by lipids has two noteworthy characteristics. It appears at low lipid concentrations (even 10 μM), and it is exerted by more than one class of long chain alkyl compound (20). It seemed desirable to delineate further this apparent lack of specificity, as well as the relative activities of different compounds. For this purpose an extensive series of alkyl compounds has been tested.

The procedure is illustrated in table II for the oleic acid derivatives. A series of dilutions for each compound over its presumed range of activity was run in two separate experiments (a and b). In this manner both the optimum concentration and the maximal section growth obtained with each compound could be estimated. Both criteria led to the following order of effectiveness: triolein > methyl oleate > monolein >> oleic acid. Triolein was equated with three times greater molarities of the other compounds as it has three oleyl residues per molecule. A comparison of its concentration with that of IAA shows that only 6 to 18 times more oleyl groups than auxin molecules are required for maximal growth. Triolein was one of the most active compounds tested.

Since only a few compounds could be tested together in a single experiment one of the most active compounds, methyl linoleate, always was included as a standard. In the course of this work, however, a paper appeared (2) which indicated that emulsions of ethyl linoleate are rapidly autoxidized in the presence of air at room temperature. Therefore, another, equally active but more stable compound, namely methyl myristate, was substituted as standard in subsequent experiments. The relative activities of different compounds, compared to these standards, are shown in table III.

These data clearly demonstrate that the most active class of compounds are the methyl and ethyl esters of the fatty acids, followed by the triglycerides. But not one of these compounds increased growth of the

Table II

Comparison of Growth Stimulating Effect of Oleic Acid Derivatives

	μM	Oleic acid		Methyl oleate		Monoolein		μM	Triolein	
Expt.		*a*	*b*	*a*	*b*	*a*	*b*		*a*	*b*
	1	...	...	...	...	...	...	0.3	...	76.1
	4	...	...	...	...	...	...	1.3	...	90.2
	10	71.2	75.8	70.1	...	68.2	61.6	3.3	101.5	99.9
	20	70.7	...	78.1	69.0	69.2	...	6.7	107.9	92.0
	30	69.3	...	107.6	95.6	88.2	...	10	110.7	...
	40	67.2	84.2	105.3	95.3	74.1	97.1	13	95.4	94.0
	50	...	...	...	92.0	98.2	...	17	98.3	...
	100	...	68.7	...	...	...	77.8	33	...	...
	300	...	75.6	...	...	...	67.7	100	...	...
						a	*b*			
				Controls		40.6	34.2			
				IAA + GA_3		74.4	70.5			

Percentage increase in length of 10 mm sections of Laxton's Progress pea epicotyls after 24 hours in 1.25 % sucrose + 50 μM $CoCl_2$ + 5 mM KH_2PO_4 (pH 5.5) + 0.003 % Pluronic F-68, and additives as listed. All but controls also 1.7 μM IAA and 0.3 μM GA_3.

sections above the values reported for methyl linoleate in table I. Since all these fatty acids are common constituents of plant fats, it can be assumed that most natural fats would be active. Accordingly, no further study has been made of the natural glyceride fraction isolated earlier (20), particularly since the behavior of this fraction in the bioassay was not different from that of the chemically better defined substances studied here.

Remarkably, the free acids have shown no consistently significant activity in any test series (the lone datum for oleic acid at 40 μM could not be repeated in other tests). But two monoglycerides, the higher alcohols, and selachyl alcohol accentuated section growth. In addition to the substances in table III, methyl or ethyl esters of the C_{10}, C_8, C_7, C_6 acids, monacetin, monopropionin, and monobutyrin, as well as acetate, triacetin, and glycerol have also been tested at various concentrations. In no case has any of these, or any other compound containing a chain of less than 12 carbon atoms, been found to exhibit action on the section growth, except to inhibit it at relatively high concentrations.

Studies of Mechanism of Action of Alkyl Lipids: A number of experiments have been run in an attempt to clarify the mechanism of action of these substances. As briefly mentioned previously (20), the activity of Tween detergents and the unsaturated fatty acid esters, as well as the inactivity of free acids, are reminiscent of the growth response of both a Lactobacillus and a Venturia mutant. But in other respects, this analogy fails. Esters of saturated fatty acids are active in the peas but not with the mutants. Conversely, biotin is active in the latter, but is inactive (0.1-50 μM) on the peas. The pH is important for the response of the mutants (but tests of the isolated pea glycerides (20) and of methyl linoleate showed that varying pH between 4.5 and 7 did not much modify the growth of the sections). It thus seems unlikely that the pea response is really analogous to that of the two microorganisms.

The smut fungus, *Ustilago zeae*, produces in culture quantities of ustilagic acid (a mixture of diglucosyl dihydroxy hexadecanoic acids) (4). The malformations characteristic of smut infection are believed to be at least partially due to its production of auxin (29). In the thought that ustilagic acid might also be involved, a sample (kindly supplied by Dr. R. H. Haskins) was tested on the pea sections. No growth promotion was noted, but inhibition was found at low levels (10 μM), with toxic effects appearing in the tissues at two to three times this concentration.

The activity of the Tween 20, 80, and 81 detergents (20) made it possible that some type of surface action is responsible for the growth promotion. However, other non-ionic surfactants, such as the Igepals CO-610, CO-630, and CO-710 (nonylphenoxypolyoxyethylenes—General Aniline and Film Corp.), and the Pluronics and Tetronics mentioned earlier, were inactive over a wide range of concentrations. An anionic wetting agent, Alrowet D-65 (sodium dioctylsulfosuccinate—Geigy Chemical Corp.), at 3 and 30 mg/l failed to stimulate growth. Some cationic detergents, Sarkosyls NL-30, O, and S (lauroyl, oleoyl, and stearoyl sarcosine—Geigy Chemical Corp.), at 3 to 300 μM were impotent or inhibitory. Carboxymethyl cellulose (CMC-70 premium low viscosity—Hercules Powder Co.), used commercially as a colloidal emulsion stabilizer, was inhibitory at concentrations greater than 0.01 % and without any

Table III

Relative Activities of Alkyl Lipids on Dwarf Pea Epicotyl Section Elongation

Alkyl group		Free acid	Ester *	Mono-glyceride **	Triglyceride	Alkyl-glyceryl ether	Alcohol
$C_{12}H_{24}$	Lauric	0	+(Me)	...	...	...	...
$C_{14}H_{28}$	Myristic	0	+++(Me)	0	...	...	0
$C_{16}H_{32}$	Palmitic	0	+++(Me, Et)	+	+	...	0
$C_{18}H_{36}$	Stearic	0	+(Et)	0	++	0 (Batyl)	+
$C_{18}H_{34}$	Oleic	0	+++(Me)	++	+++	+++(Selachyl)	...
$C_{18}H_{32}$	Linoleic	0	+++(Me)	...	+++	...	...
$C_{18}H_{30}$	Linolenic	...	++(Me)	...	...	...	...
$C_{20}H_{40}$	Arachidic	...	...	0	...	...	++
$C_{20}H_{32}$	Arachidonic	...	+++(Me)	...	...	...	...
$C_{22}H_{44}$	Behenic	...	...	0	...	...	++
$C_{22}H_{42}$	Erucic	...	...	...	...	...	++
$C_{18}H_{32}$	Brassidic	0	...	...	...	...	...
	Chaulmoogric	...	++(Et)	...	...	...	...

Basal medium as in table II, including IAA and GA_3. Relative activities are compared to maximum growth promotion over controls obtained with methyl myristate or methyl linoleate on the same day: 0 = less than 20 % of maximum promotion; + = from 20 to 50 % of maximum promotion; ++ = from 50 to 80 % of maximum promotion; +++ = same as (± 20 %) maximum promotion.

* Me = methyl ester and Et = ethyl ester of corresponding acid.

** Kindly supplied by Distillation Product Industries.

action at lower percentages. Furthermore, the large number of active compounds in table III do not seem to have any obvious common physical property which would not be shared by some of the inactive substances. Surface action thus appears to be an untenable explanation unless it is exerted inside the cell after some metabolic modification of the molecules.

The next most likely explanation of the growth promoting effect of the lipids was considered to be an action on the cell membrane. The site of auxin action is believed to be on the cell wall or membrane, and furthermore, auxins are known to increase cell permeability (25). As phospholipids are constituents of membranes, it is possible that the lipids are entering into their metabolism and in some way promoting auxin action. Bennet-Clark has proposed an hypothetical auxin mechanism involving phospholipids (3). The metabolic sequences thought to be involved in phospholipid synthesis have been summarized by Kennedy (15). Of the various cofactors he cites, choline (0.4, 4, 40 μM), phosphoryl choline (10, 30, 100, 300, 10^4 μM), and cytidine (5, 50 μM) were all found to be ineffective on the pea sections, as were uridine (5, 50 μM) and uracil (5, 50, 100 μM). A characteristic requirement of phospholipid synthesis is that both saturated and unsaturated fatty acids are needed. When mixtures of methyl palmitate and methyl linoleate were tested on our system, however, their maximal effect on section extension was no greater than that of either compound alone. At submaximal concentrations simple additivity of their action was indicated. A phospholipid, lecithin (0.0003-0.003 %), was also inactive.

If the cell membrane is involved it might also be that the entry of auxin into the cell is facilitated. Some crude tests with C^{14}-carboxyl-labeled IAA have been carried out, but did not show any convincing increase in the disappearance of IAA from the external solution in the presence of lipid factors as compared to non-lipid-treated controls. Thus, no evidence implicating an action of the growth-promoting lipids on the membrane has yet been found.

Another group of long carbon chain compounds found in plants is the phytosphingolipids containing phytosphingosine. Their role in plant metabolism is obscure, but Carter et al (7) believe that they also contain inositol, hexuronic acids (probably galacturonic acid), glucosamine, arabinose, galactose, and mannose. These compounds at 0.01 and 0.1 % were accordingly tested both in the presence and absence of methyl myristate, but no further promotion of section growth was noted. Phytosphingosine (10-50 μM), generously supplied by Professor Carter, was similarly without effect.

Finally, the possibility that these lipids might be acting via the conventional degradative pathway for fatty acids was examined in several ways. The action of cobalt and sucrose in promoting section growth (18, 24) is not understood, and since pea sections are rich in stored materials (8), it seemed possible that sucrose was acting as a remote source of lipid materials via fatty acid synthesis. Figure 1 shows a test of this hypothesis. It demonstrates that the lipid response is almost completely dependent on added sucrose and hence the action of sucrose cannot primarily be as a substitute for alkyl lipids. Also, as noted above, acetate and glycerol were inactive. So was sodium pyruvate over a concentration range of 0.03 to 3 mM. Marrè and Arrigoni (17) have reported slight promoting effects of reduced glutathione on pea sections under different conditions than ours. In our bioassay, cysteine (3 and 10 mg/l), reduced glutathione (0.1, 0.3, 1 mM) and thioctic acid (5, 20, 60 μM) failed to accelerate section growth either in the presence or absence of lipid. Equally ineffective were coenzyme A (5, 20, 60 μM) and pantothenic acid (1, 10, 100 μM).

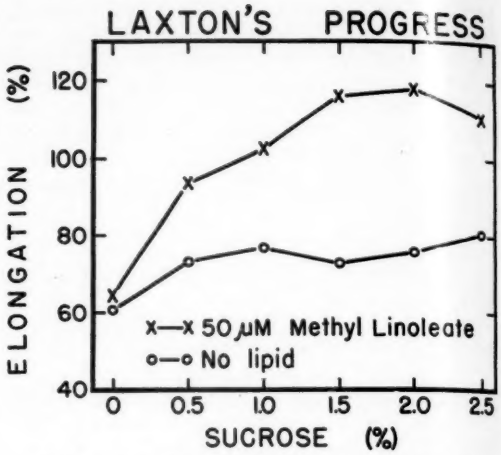

FIG. 1. The effect of sucrose on the growth of dwarf pea epicotyl sections and its promotion of the growth stimulation caused by a lipid. All sections treated with 1.7 μM IAA and 0.3 μM GA_3 and basal medium as in table I.

The action of many lipid-soluble substances on pea sections seems at first sight similar to the wound hormone factors reinvestigated recently by Haagen-Smit and Viglierchio (12). When a detailed comparison was made, it was the differences between our system and the Wehnelt bean test they used which became really striking. First, traumatic acid (10) was inactive on peas (4-130 μM). Next, these workers found the most active substances to be free fatty acids, including lauric, myristic, and linoleic acids. In our tests these substances were not active at all. Finally, the wound response of the bean pods to linolenic acid was greatly promoted by cytochrome c and ascorbic acid. In our tests horse heart cytochrome c (0.03-3 μM) and ascorbic acid (3 μM) added to the pea sections both singly and together, as well as in the presence of methyl myristate (30 μM),

were ineffective. Cytochrome c did cause moderate inhibition at the higher concentrations. None the less, the fact that results of Haagen-Smith and Viglierchio also show an apparently hormonal response to long-chain alkyl substances at concentrations lower than 200 μM suggests that bean plaques and pea sections share some common hormonal mechanism.

Discussion

While this work was in progress Vlitos and Crosby (26) isolated a higher alkyl alcohol from tobacco and showed it to be active in the Avena first internode auxin assay, as were several other alkyl compounds. Struckmeyer and Roberts (23) have also found a higher alcohol in plant extracts and believe it interacts with auxin. These discoveries, when compared with the data of Haagen-Smit and Viglierchio (12), the earlier work on wound hormones (10) and that of this paper, make it clear that trace amounts of lipid substances do play some role in the hormonal physiology of plants. These substances cannot be said to be limiting in all auxin bioassay material, however, as our tests have so far failed to show any effects on *Avena* coleoptiles (20).

Further support for our conclusion that lipids are involved in plant growth regulation can be found in studies of the use of auxin sprays for horticultural and herbicidal purposes. As shown by Harley et al (13), M. N. Westwood (private communication), and Hitchcock and Zimmerman (14), the efficiency of such sprays can be increased by adding Tween 20. This phenomenon has been interpreted as due to increased wetting or penetration of the spray. Harley et al (13), however, noted that Tween 20 was a weak thinning agent in itself. And recent work by L. L. Jansen (private communication) indicates that a number of commercial detergents can by themselves increase the growth of several intact plants. It would be interesting to compare the effectiveness of alkyl with non-alkyl detergents in tests such as these.

A link between lipid substances and auxin action has also been indicated by the work of Christiansen and Thimann (8) who showed that the ether-soluble fraction of pea stem sections dropped during growth. More significantly, this drop was reduced by the same inhibitors that reduced cell elongation. These workers, in summarizing previous work as well as their own, concluded that some interaction between fat and reducing sugar levels was indicated. The present work shows that sugars are needed for lipids to enhance growth. The added fats cannot be a quantitatively important source of sugars here since nearly all the alkyl lipids tested are most effective in the range of 10 to 50 μM. This is only 6 to 30 times the concentration of IAA which was employed in the tests: it suggests that a hormonal rather than a nutritive requirement is involved. Inasmuch as it was necessary to employ emulsions of these lipids the actual molar ratios could even be lower and approach mole to mole values.

The data of Christiansen and Thimann show further that during growth only a third of the neutral ether-solubles are utilized. The amount still left in the section is five times more than would be required to produce the effects discussed in this paper. Therefore, since nearly all normal fat constituents have activity in the bioassay, it appears that not all lipids present in the tissue are available to take part in the synergism. However, sections were not grown by Christiansen and Thimann in the presence of added sucrose so that their analyses are not strictly comparable. It will be necessary to analyze lipid metabolism in pea sections and in the intact plant in detail before any conclusions can be made concerning the natural importance of the synergism of lipids with plant hormones.

Neither can any firm conclusions yet be drawn as to the role these lipids are playing in plant metabolism. The tests described in this paper, although limited to the bioassay technique, have given no support to the idea that well known biochemical pathways of lipids are involved. The most promising explanation developed so far has come out of further work which indicates that these factors may be activating the cytochrome system; the preliminary evidence for this has been outlined elsewhere (21).

This work also makes it clear that some unexpected factor other than lipids is involved in section growth. Dwarf peas were employed because it was anticipated that they would be more sensitive to gibberellin (5). Yet in every instance their sections gave a response remarkably similar to that of Alaska pea sections (see table I in this paper and figure 1 in 20). This amplifies the results of von Abrams (1) who could not find appreciable differences between a dwarf and a standard pea when several aspects of auxin relations were considered. Hence it appears that the striking effect of gibberellic acid on dwarf peas discovered by Brian and Hemming (5) is still in some way restricted to intact plants. The data of Lockhart (16) and Galston and Warburg (11) also suggest that the ineffectiveness of gibberellic acid on sections may be due to the lack of a factor produced by the rest of the plant, as a greater response was shown by decapitated sections left attached to the base of the plant. Our attempts to extract the factor indicated by such experiments have not as yet been successful. Comparing these papers with the older publication of Went (28) shows that the factor gibberellin action requires may well be the "caulocaline" which Went showed to be produced by roots and which only passed through living tissue. The appearance of the caulocaline effect required pea tip auxin, but as Lockhart (16) has shown, pea tips also produce gibberellin.

But a completely different explanation based on the results of Phinney and Neely (19) cannot yet be excluded. They found that dwarf mutants of maize vary in their response to different gibberellins, and also found that one mutant (dwarf-1) was much less responsive than the others to a natural gibberellin, bean factor II (GA_5), isolated from a higher plant.

They have advanced the hypothesis that this indicates a biochemical pathway of gibberellin synthesis. Therefore, it may be that GA_3 (and the GA_1 in the mixture employed by Lockhart) are not active on growth of peas in themselves, but must undergo some alteration before they participate in the growth process. Thus the relative inactivity of GA_3 on pea sections may merely mean that the sections do not possess the enzymes necessary to convert it to an active form and that these enzymes are elsewhere in the plant. In any case, further analysis of the puzzling difference between the growth of intact plants and excised sections seems certain to cast more light on the factors involved in natural growth regulation.

Summary

A technique is described for preparing stable aqueous emulsions of lipids, allowing their routine use in bioassays.

Excised sections of both dwarf and Alaska peas, even under optimal conditions, fail to attain half the growth they would have reached in situ on an intact plant.

Part of this missing growth can be restored by many lipid substances, especially by several types of fatty acid esters. The specificity of this response is delineated. The most effective compounds found so far are: methyl myristate, methyl palmitate, methyl oleate, methyl linoleate, methyl arachidonate, triolein, trilinolein, and selachyl alcohol. At their optima, these materials can bring the growth of sections up to just half that upon the intact plant.

To observe the growth promoting activity of these lipids, it is necessary that sugar and auxin be supplied in the medium. Their action is enhanced further by gibberellic acid.

Tests of various cofactors of lipid metabolism have failed to establish a connection with the growth effect shown by the alkyl lipid compounds. This effect seems most likely to be hormonal in nature since the amounts of lipid required are comparable to that of the auxin used.

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Literature Cited

1. Von Abrams, G. J. 1953. Auxin relations of a dwarf pea. Plant Physiol. 28: 443–456.
2. Bayzer, H., J. Biheller, H. Gaisch, and E. Schauenstein 1958. Über die Reaktion von 9,12-Linolsäureäthylester in Wasser. Monatsh. Chem. 89: 258–269.
3. Bennet-Clark, T. A. 1956. Salt accumulation and mode of action of auxin. A preliminary hypothesis. In: The Chemistry and Mode of Action of Plant Growth Substances, R. Wain and F. Wightman, eds. Pp. 284–291. Butterworths, London.
4. Boothroyd, B., J. A. Thorn, and R. H. Haskins 1955. The biochemistry of the Ustilaginales. X. The biochemistry of ustilagic acid. Can. Jour. Biochem. Physiol. 33: 289–296.
5. Brian, P. W. and H. G. Hemming 1955. The effect of gibberellic acid on shoot growth of pea seedlings. Physiol. Plantarum 8: 669–681.
6. Brian, P. W. and H. G. Hemming 1958. Complementary action of gibberellic acid and auxins in pea internode extension. Ann. Bot. 22: 1–17.
7. Carter, H. E., D. S. Galanos, R. H. Gigg, J. H. Law, T. Nakayama, D. B. Smith, and E. J. Weber 1957. Sphingolipides. Federation Proc. 16: 817–824.
8. Christiansen, G. S. and K. V. Thimann 1950. The metabolism of stem tissue during growth and its inhibition. II. Respiration and ether-soluble material. Arch. Biochem. 26: 248–259.
9. Denny, F. E. 1938–1939. Leaf epinasty tests with chemical vapors. Contrib. Boyce Thompson Inst. 10: 191–195.
10. English, J. E., Jr., J. Bonner, and A. J. Haagen-Smit 1939. The wound hormones of plants. IV. The structure and synthesis of a traumatin. Jour. Amer. Chem. Soc. 61: 3434–3436.
11. Galston, A. W. and H. Warburg 1959. An analysis of auxin-gibberellin interaction in pea stem tissue. Plant Physiol. 34: 16–22.
12. Haagen-Smith, A. J. and D. R. Viglierchio 1955. Investigation of plant wound hormones. Rec. trav. chim. 74: 1197–1206.
13. Harley, C. P., H. H. Moon, and L. O. Regeimbal 1957. Effects of the additive Tween 20 and relatively low temperatures on apple thinning by naphthalene-acetic acid sprays. Proc. Amer. Soc. Hort. Sci. 69: 21–27.
14. Hitchcock, A. E. and P. W. Zimmerman 1948. Activation of 2,4-D by various adjuvants. Contrib. Boyce Thompson Inst. 15: 173–193.
15. Kennedy, E. P. 1957. Metabolism of lipides. Ann Rev. Biochem. 26: 119–148.
16. Lockhart, J. A. 1957. Studies on the organ of production of the natural gibberellin factor in higher plants. Plant Physiol. 32: 204–207.
17. Marrè, E. and O. Arrigoni 1957. Metabolic reactions to auxin. I. The effects of auxin on glutathione and the effects of glutathione on growth of isolated plant parts. Physiol. Plantarum 10: 289–301.
18. Miller, C. O. 1954. The influence of cobalt and sugars upon the elongation of etiolated pea stem segments. Plant Physiol. 29: 79–82.
19. Ph'nney, B. O. and P. M. Neely 1958. Differential biological properties of gibberellin-like factors isolated from beans and peas. Plant Physiol. 33 suppl.: xxxviii.
20. Stowe, B. B. 1958. Growth promotion in pea epicotyl sections by fatty acid esters. Science 128: 421–423.
21. Stowe, B. B. 1959. Similar activating effects of lipids on cytochromes and on plant hormones. Biochem. Biophys. Res. Comm. 1: 86–90.

22. STOWE, B. B. and T. YAMAKI 1959. Gibberellins: Stimulants of plant growth. Science 129: 807–816.
23. STRUCKMEYER, B. E. and R. H. ROBERTS 1955. The inhibition of abnormal cell proliferation with anti-auxin. Amer. Jour. Bot. 42: 401–405.
24. THIMANN, K. V. 1956. Studies on the growth and inhibition of isolated plant parts. V. The effects of cobalt and other metals. Amer. Jour. Bot. 43: 241–250.
25. THIMANN, K. V. and E. W. SAMUEL 1955. The permeability of potato tissue to water. Proc. Nat. Acad. Sci., U.S. 41: 1029–1033.
26. VLITOS, A. J. and D. G. CROSBY 1959. Isolation of fatty alcohols with plant-growth promoting activity from Maryland Mammoth tobacco. Nature 184: 462–463.
27. WÄCHTER, W. 1905. Chemonastische Bewegungen der Blätter von *Callisia repens*. Ber. deut. bot. Ges. 23: 379–382.
28. WENT, F. W. 1938. Specific factors other than auxin affecting growth and root formation. Plant Physiol. 13: 55–80.
29. WOLF, F. T. 1952. The production of indoleacetic acid by *Ustilago zeae*, and its possible significance in tumor formation. Proc. Nat. Acad. Sci., U.S. 38: 106–111.

BIOSYNTHESIS OF SUCROSE AND SUCROSE-PHOSPHATE BY SUGAR BEET LEAF EXTRACTS[1]

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Cardini and coworkers (6) reported as one part of a plant materials survey that "negative or non-reproducible results" were obtained in sucrose synthesis studies with sugar beet leaves and roots. They stated that these results "may be attributed to the presence of interfering enzymes".

Burma and Mortimer (5), using isotopic tracer techniques, reported the synthesis of sucrose by sugar beet leaf homogenates when fructose-6-phosphate and uridine diphosphoglucose (UDPG) were present. They demonstrated that sucrose-phosphate was formed and assumed that it was subsequently dephosphorylated by phosphatase to sucrose. No sucrose synthesis occurred when fructose or fructose-1,6-diphosphate and UDPG were added to sugar beet leaf homogenates. Burma and Mortimer stated that their experiments with sugar beet leaves eliminated Leloir and Cardini's (11) first enzyme mechanism demonstrated with wheat germ, which was the interaction of UDPG and fructose for the direct formation of sucrose plus pyrophosphate.

This paper presents data showing that the assumption of Burma and Mortimer that only sucrose-phosphate is synthesized directly does not seem to be correct. Rather, it shows that sugar beet leaf tissue contains enzyme systems for the direct synthesis of sucrose as well as sucrose-phosphate through UDPG and fructose and UDPG and fructose-6-phosphate, respectively.

Any sucrose-phosphate which is formed is ultimately dephosphorylated by enzymes in sugar beet tissue at some stage prior to storage in the root. Thus the synthesis of both sucrose and sucrose-phosphate also has some bearing on the important unresolved question of whether sucrose or sucrose-phosphate is the principal sugar transported to the root for storage (4, 8, 9, 10, 18).

MATERIALS AND METHODS

Preliminary experimentation resulted in adopting the following procedures for preparing beet leaf enzyme fractions active in the synthesis of sucrose and sucrose-phosphate. All operations were carried out at 0 to 5° C.

Six hundred grams of fresh leaves of 6 to 7 week old sugar beet seedlings (*Beta vulgaris* var GW 304) were used for preparing the enzyme fractions. A 100 g aliquot of seedling leaves was blended with 100 ml of 0.05 M, pH 7.2 phosphate buffer in a high speed blender. This homogenate was then squeezed in a fine nylon cloth. The liquid obtained was used for blending the next 100 g aliquot of leaves. This process was repeated until all 600 g of leaves had been homogenized, using only the original 100 ml of phosphate buffer diluent. The pH was periodically adjusted to 6.8 to 7.0 with NaOH during the blending procedure. Approximately 600 ml of filtered homogenate was obtained. This was centrifuged at 13,000 × G, and the precipitate obtained was discarded. The supernatant was gradually taken to pH 4.9 with acetic acid and immediately centrifuged at 13,000 × G for 15 minutes. The precipitate was discarded and the clear yellowish supernatant was left overnight at

[1] Received August 3, 1959.

[2] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

4° C. The flocculent precipitate which then formed was collected by centrifugation and washed several times with 0.02 M, pH 4.9 acetate buffer. The recentrifuged precipitate was then dissolved in sufficient 0.05 M, pH 7.2 phosphate buffer to make a thick slurry, and this final fraction was then dialyzed overnight in a continuous dialyzer against 0.02 M, pH 7.2 phosphate buffer. This enzyme fraction was used throughout these studies.

The complete reaction mixture for sucrose synthesis studies contained 1.5 μM UDPG, 4 μM fructose, 0.1 ml enzyme fraction (containing 1.4 mg protein nitrogen), 0.002 ml 0.1 M $MgCl_2$, and 0.01 ml of 1 M Tris buffer. If a pH above 9.0 was desired, ethanolamine-HCl buffer was substituted for the Tris buffer. The total volume of this reaction mixture was 0.2 ml. The complete reaction mixture for the study of sucrose-phosphate synthesis was identical to the mixture used for sucrose synthesis except that 4 μM of fructose-6-phosphate was substituted for the fructose and in addition 0.01 ml of 1 M KF was present as a phosphatase inhibitor. After 4 hours incubation at 37° C, the tubes containing the enzyme and substrates were placed in a boiling water bath for 5 minutes and then cooled. To each tube was added 0.8 ml of a solution of 0.025 *N* NaOH containing 15 mg $NaBH_4$. This was followed by adding a few drops of ethanol as a foam retardant. These tubes were left for 1 hour at room temperature and were then covered with a glass marble and placed in a boiling-water bath for 5 minutes to complete the reduction of the hexoses to their corresponding sugar alcohols. The use of NaOH only and heating to 100° C to eliminate reducing sugars (6) gave an excessive discoloration which was not observed in the borohydride reduction reaction. The reproducibility and sensitivity of the subsequent resorcinol procedure of Roe (17) for determinations of sucrose was greatly improved by using the borohydride procedure to eliminate fructose and glucose. It was necessary, however, to destroy the excess of borohydride by acidifying the mixture with a few drops of acetic acid before proceeding with the resorcinol method. For Roe's procedure, the volumes were reduced to half and the color was measured in a 1 cm cell at 490 mμ in a Beckman Model B spectrophotometer. [3]

The biosynthesis of sucrose and sucrose-phosphate was confirmed by paper chromatography of aliquots of the inactivated enzyme reaction mixture following passage of the mixture through a cold mixed bed ion exchange column containing equal amounts of Dowex-50 (H^+ form) and Duolite A-4 (OH^- form) resins. Sucrose-phosphate remained on the column. Pretreatment at pH 9.7 with invertase-free alkaline phosphatase dephosphorylated sucrose-phosphate and produced sucrose in the column eluate. Aliquots of the ion-exchanged solution were concentrated, spotted, and developed on papers in a solvent of ethyl acetate, pyridine, and water (8:2:1 v/v) (19). Some chromatograms were developed in a solvent of *n*-propanol, ethyl acetate, and water (7:1:2 v/v) (1). Fructose and sucrose spots were detected on the papers by a dip of 0.5 % resorcinol in ethyl acetate made 0.2 *N* with concentrated HCl, followed by air drying and heating at 85° C for 5 minutes.

The enzyme fraction used for the sucrose and sucrose-phosphate synthesis studies was assayed for invertase activity in a mixture consisting of 0.01 ml pH 7.0 Tris buffer, 30 μM sucrose, 0.003 ml 0.1 *N* $MgCl_2$ and 0.3 ml of enzyme (4.2 mg of protein N) in a total volume of 1 ml.

This enzyme fraction was also assayed for phosphatase activity at pH 7.0 in a mixture identical to that used for the invertase assay, except that 10 mg of fructose-6-phosphate was substituted for the sucrose. Inorganic phosphate resulting from hydrolysis of the fructose-6-phosphate was determined colorimetrically by a modification of the method of Allen (2) in which the samples were diluted to 15 to 18 ml with water before adding perchloric acid, amidol reagent, and molybdate. The tubes were read at 660 mμ after 10, but not more than 20 minutes, and were compared with known standards.

Results and Discussion

Assays of the pH 4.9 precipitated enzyme fraction revealed that some invertase was still present. After 2 hours incubation at pH 7.0, 0.3 ml of the enzyme inverted 5 and 10 μM of sucrose in 2 and 4 hours, respectively. Additional studies with this enzyme fraction at pH 9.0 revealed a 40-fold decrease in invertase activity from that found at pH 5.0. The described purification procedures decreased the amount of invertase present in the enzyme fraction to a level where detectable amounts of sucrose could now be accumulated. Studies of pH effect on sucrose synthesis with our enzyme fraction are shown in figure 1. Optimum sucrose synthesis occurred at pH 8.7 and then decreased sharply up to pH 9.7. This does not mean that the pH optimum of the uridine diphosphoglucose fructose transglycosylase is 8.7, but that at this pH the rate of the transglycosylase exceeds the action of invertase by the greatest amount. One enzyme preparation gave optimum sucrose synthesis at pH 8.4. This may have been due to the presence of a lesser amount of invertase. Other small variations were also found on replication of the enzyme fractionation procedure. In several cases it was difficult to get a good precipitate after leaving the yellowish supernatant overnight at pH 4.9. More consistent precipitates, but of lower activity for sucrose synthesis, were obtained if the yellowish supernatant was dialyzed against distilled water prior to the adjustment to pH 4.9. When only 100 ml of buffer was used to homogenize 600 g of leaf tissue, an adequate

[3] Mention of specific products does not constitute endorsement by the Department of Agriculture over others of a similar nature not mentioned.

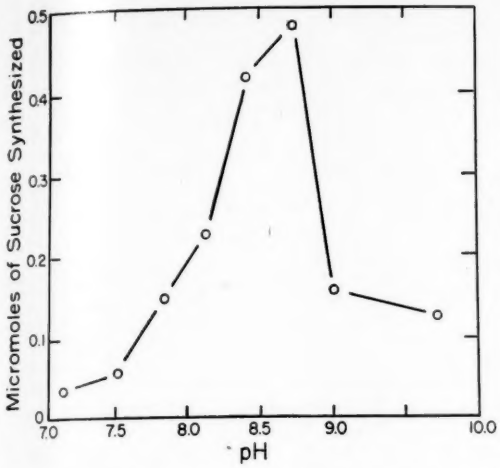

FIG. 1. The effect of varying pH on the synthesis of sucrose by the sugar beet leaf enzyme preparation.

precipitate of active protein following pH adjustment was usually obtained.

This enzyme fraction was active in both sucrose and sucrose-phosphate synthesis. Under the described assay conditions, from 0.15 to 0.25 micromoles of sucrose-phosphate were synthesized. Optimum sucrose-phosphate synthesis was observed when 0.01 to 0.03 ml of 1 M KF was present as a phosphatase inhibitor. The same amount of KF inhibited sucrose synthesis by approximately 25 % at pH 8.7. Ammonium molybdate markedly inhibited sucrose-phosphate synthesis when used as a phosphatase inhibitor at a concentration of 0.001 M.

Phosphatase activity observed under the described assay conditions was fairly low, with less than 10 % of the fructose-6-phosphate present being dephosphorylated even after 4 hours incubation.

The identities of sucrose and sucrose-phosphate were verified in several ways. When the enzyme-substrate mixture used for sucrose synthesis was passed through the mixed bed ion exchange column and spotted on paper, a neutral substance moving with an R_f identical to that of sucrose was found to have passed through the column. Chromatography of the material in this spot after treatment with analytical invertase resulted in spots which moved identically with known glucose and fructose. The incubation mixture before passage through the ion exchange column and the eluate of the column contained the same amounts of sucrose as determined by the resorcinol procedure of Roe (17).

Sucrose-phosphate synthesized by the incubation mixture was adsorbed on the mixed bed column. A negative resorcinol test was obtained with the eluate. A positive test for sucrose was observed in the eluate when the heat-inactivated incubation mixture containing sucrose-phosphate was taken to pH 9.7, treated with alkaline phosphatase and then passed through the mixed bed column.

Studies with *Canna indica* leaves (16) demonstrated that when labeled glucose was administered to leaf discs, the percentage of total activity in the glucose decreased while the activity of sucrose showed a parallel increase. No activity was detected in the free fructose, but both moieties of sucrose were found to have been labeled. Infiltration of labeled fructose to the leaf discs resulted in a similar C^{14} labeling pattern of the sucrose, but free glucose was not labeled. The same experiments of Putman and Hassid (16) also gave very strong evidence for the existence of the endogenous free hexoses in inert pools. Edelman, Ginsburg, and Hassid (7) state that glucose and fructose taken up by Canna leaves and wheat seedlings may become phosphorylated at the time of entry into the leaf tissue and that these sugars do not enter the monosaccharide compartments of the plant. These workers obtained results with wheat seedlings which were in agreement with the Canna leaf disk studies. Sucrose synthesized following introduction of labeled glucose was labeled in both moieties but free labeled fructose did not appear. However, the participtation of free fructose in sucrose synthesis cannot be ruled out on the basis of these experiments. Leloir and Cardini (12) state that studies on green leaves with labeled substrates would prove that free fructose is not involved directly in the synthesis of sucrose except for the fact that the same experiments demonstrated storage of sugars in leaves in metabolically inert compartments. In other words, administered glucose and fructose do not equilibrate with the corresponding endogenous pools of these sugars and thus cannot be considered as metabolically equivalent to the endogenous stored glucose and fructose. When glucose is administered, the labeling rate of the fructose and its incorporation into sucrose at the site of sucrose synthesis is probably much faster than labeling fructose and its transport into the metabolic pools. The labeling experiments have demonstrated only that administered free hexoses do not enter the endogenous free hexose pools and have not shown, conversely, that the hexoses of the pools cannot participate, either free or through subsequent phosphorylation, in sucrose synthesis. In sugar beet leaf tissue, the endogenous sucrose and fructose pools have been reported as 1.7 and 4.7 mg, respectively, per gram of fresh leaf tissue (13). Pavlinova (15) has reported similar values for these pools. Because of the small amounts of hexose phosphates in sugar beet leaf (14) and the fact that one would expect these sugar phosphates to be in a state of equilibrium since phosphohexoisomerase is present and active, it is logical to assume that following the introduction of either labeled glucose, fructose, or of CO_2, any deviation from equal labeling of the hexose moieties constitutes fair evidence that the route of sucrose synthesis is not entirely through phosphorylated fructose. There are several examples of a significant disparity of label of the sucrose hexoses, including the studies of Axelrod and Seegmiller (3) with apple tissue and those of Putman

and Hassid (16) with Canna indica. Nelson and Mortimer (13) found that the specific activity of the fructose moiety of sucrose was lower than that of the glucose moiety when excised sugar beet leaves were killed after carrying on photosynthesis in the presence of $C^{14}O_2$ followed by $C^{12}O_2$ and this labeling pattern was interpreted on the assumption that sucrose can be formed in sugar beet leaves only from UDPG and fructose-6-phosphate. In earlier studies, Burma and Mortimer (5) reported that following the introduction of labeled glucose-1-phosphate to a sugar beet leaf, the resulting sucrose on inversion had "approximately equal activities in both glucose and fructose."

Even if the label of sucrose in a plant tissue is found to be equal in the two hexose moieties, one still cannot rule out the participation of free fructose in sucrose synthesis because of the possibility of dephosphorylation of fructose-6-phosphate at the site of synthesis. It seems impossible to assess the degree of participation in sucrose synthesis of either fructose or fructose-6-phosphate on the basis of only the final labeling of sucrose in leaf or whole plant experiments.

Summary

It was demonstrated that sugar beet leaf tissue contains active systems for the synthesis of both sucrose and sucrose-phosphate as indicated below. This is the first time that reaction I has been shown to be operative in green leaf tissue.

I. UDPG + fructose ⟶ sucrose + UDP
II. UDPG + fructose-6-phosphate ⟶ sucrose-phosphate + UDP

Methods for the purification and assay of the enzyme fraction are described as are conditions for demonstrating its maximum activity in the presence of invertase. All attempts to prepare enzyme fractions from sugar beet leaves which were free of invertase failed. Methods are described for the differentiation of sucrose and sucrose-phosphate formed in enzyme-substrate incubation mixtures.

Acknowledgements

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Literature Cited

1. Albon, N. and D. Gross 1952. The chromatographic determination of raffinose in raw sugars. Analyst 77: 410–412.
2. Allen, R. J. L. 1940. The estimation of phosphorus. Biochem. Jour. 34: 858–865.
3. Axelrod, B. and C. G. Seegmiller 1954. Metabolism of apples. Conversion of glucose to sucrose in apple tissue. Jour. Agr. and Food Chem. 2: 1329–1331.
4. Bougy, E. 1956. Esters phosphoriques et sucres au cours de la végétation de la Betterave. Compt. rend. 242: 667–669.
5. Burma, D. P. and D. C. Mcrtimer 1956. The biosynthesis of uridine diphosphate glucose and sucrose in sugar beet leaf. Arch. Biochem. Biophys. 62: 16–28.
6. Cardini, C. E., L. F. Leloir, and J. Chiriboga 1955. The biosynthesis of sucrose. Jour. Biol. Chem. 214: 149–155.
7. Edelman, J., V. Ginsburg, and W. Z. Hassid 1955. Conversion of monosaccharides to sucrose and cellulose in wheat seedlings. Jour. Biol. Chem. 213: 843–854.
8. Kursanov, A. L. 1954. Synthese und anhäufung von saccharose in der zuckerrübe. Botan. Zhur. S.S.S.R. 39: 482–487. (Translated in Zeit. Zuckerind. 83: 78–80. 1958.)
9. Kursanov, A. L. and M. V. Turkina 1954. Forms of mobile sugars in the conducting system of the sugar beet. Doklady Akad. Nauk SSSR 95: 885–888. (Cited in Chemical Abstracts 8881g. 1954.)
10. Kursanov, A. L., M. V. Turkina, and I. M. Dubinina 1953. The use of the isotope method in the study of movement of sugars in plants. Doklady Akad. Nauk SSSR 93: 1115–1118. (Cited in Chemical Abstracts 4642 c. 1954.)
11. Leloir, L. F. and C. E. Cardini 1953. The biosynthesis of sucrose. Jour. Amer. Chem. Soc. 75: 6084.
12. Leloir, L. F. and C. E. Cardini 1955. The biosynthesis of sucrose phosphate. Jour. Biol. Chem. 214: 157–165.
13. Nelson, C. D. and D. C. Mortimer 1959. The preparation of sucrose-C^{14} with high specific activity and high tracer yield using detached sugar beet leaves. Canadian Jour. Biochem. Physiol. 37: 193–197.
14. Pavlinova, O. A. 1952. Phosphoric esters of sugar beet. Doklady Akad. Nauk SSSR 83: 597–600. (Cited in Chemical Abstracts 9668c. 1952.)
15. Pavlinova, O. A. 1957. Use of anthrone for quantitative determinations of sugars separated on chromatograms. Plant Physiol. (A.I.B.S. translation of Fiziologia Rasteny) 4: 97–102.
16. Putman, E. W. and W. Z. Hassid 1954. Sugar transformations in leaves *of Canna indica*. I. Synthesis and inversion of sucrose. Jour. Biol. Chem. 207: 885–902.
17. Roe, J. H. 1934. A colorimetric method for the determination of fructose in blood and urine. Jour. Biol. Chem. 107: 15–22.
18. Turkina, M. V. 1954. On physiological-biological investigation of fibrovascular bundles in relation to the movement of sugars in plants. Thesis, Institute of Plant Physiology, Acad. Sci., U.S.S.R. Cited by A. A. Prokofyev and A. M. Sobolev. In: Plant Physiol. (A.I.B.S. translation of Fiziologia Rasteny) 4: 12–20. 1957.
19. Williams, K. T. and A. Bevenue 1951. The chromatographic examination of sugars in wheat flour. Cereal Chem. 28: 416–423.

SOME ANATOMICAL EFFECTS OF KINETIN AND RED LIGHT ON DISKS OF BEAN LEAVES [1]

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The application of kinetin (6-furfurylaminopurine) produces some effects similar to those induced by exposure to red light (Miller, 3, and Scott and Liverman, 5). Both cause increased expansion of disks from bean leaves, greater elongation in stems and petioles of bean seedlings, and increased germination of lettuce seeds in the dark. Both treatments inhibit internodal elongation in excised peas. Because of the striking resemblance in these effects, Miller (3) suggested that these two treatments may operate through the same biological mechanism. A subsequent study of the germination of lettuce seeds in the dark indicated that kinetin is not involved directly in the photoreaction or in the reactions which immediately precede or follow it (Miller, 4). Scott and Liverman (5) also doubted if kinetin controlled the same reaction step as red light. The objective of the present investigation was to determine if anatomical differences occur between samples of disks from bean leaves treated with kinetin and those exposed to red light.

MATERIALS AND METHODS

Disks, 7.0 mm in diameter, were cut from the first foliage leaves of 8-day-old bean seedlings, *Phaseolus vulgaris* L. (Burpee stringless green pod), which had been grown in the dark. Four groups of ten disks each were placed in Petri dishes on Whatman's #3 filter paper, and 7.0 ml of nutrient solution prepared according to a formula described by Miller (3) was added. One group of disks served as a control; two groups had different concentrations of kinetin added to the nutrient solution; the fourth was exposed to red light for 12 minutes. Red light was filtered from two 15 watt fluorescent bulbs through two sheets of Du Pont 300 MCS red cellophane. The light source was placed 30 cm from the leaf disks. Manipulations were made under a Wratten Series 3 (green) safelight, and temperature was maintained at 25° C. At the end of 48 hours, the increase in the size of the diameters of the disks was measured with the aid of an eyepiece micrometer in a dissecting microscope. An analysis of variance was made and Duncan's multiple range test (1) was applied.

The second experiment was undertaken after the results of the first were known; the same methods were employed in both experiments, but the leaf disks were cut 5.0 mm in diameter. One group served as the control. Two groups were exposed to red light; the first for 5 minutes, and the other for 10 minutes. Kinetin was added to the nutrient medium of the fourth group. The fifth group of disks was supplied with kinetin and also exposed to 5 minutes of red light. At the end of 48 hours all disks were measured and prepared for histological study.

All disks were killed and fixed in Randolph's solution, dehydrated through a tertiary butyl alcohol series, and embedded in paraffin. Sections were cut 8 μ thick with a rotary microtome and stained with tannic acid-ferric chloride and safranin. The width of the palisade cells was measured with an eyepiece micrometer.

RESULTS

Disks treated with kinetin and those exposed to 12 minutes of red light were larger in diameter after 48 hours than those of the control (table I). The difference in effect of the two concentrations of kinetin on disk size was significant at the 5 % level.

Table I shows a comparison of the average width of the palisade cells in disks at the conclusion of the first experiment. Palisade cells are wider in both of the kinetin-treated samples than in either the control or those irradiated with red light. The greater increase in width of the palisade cells from treated leaf disks is evident in the photographs. Width of the palisade cells in the disks of the control (fig 1) and in those exposed to red light (fig 3) did not differ significantly. Palisade cells in the kinetin-treated disks (fig 2) were wider, and the disks were thicker,

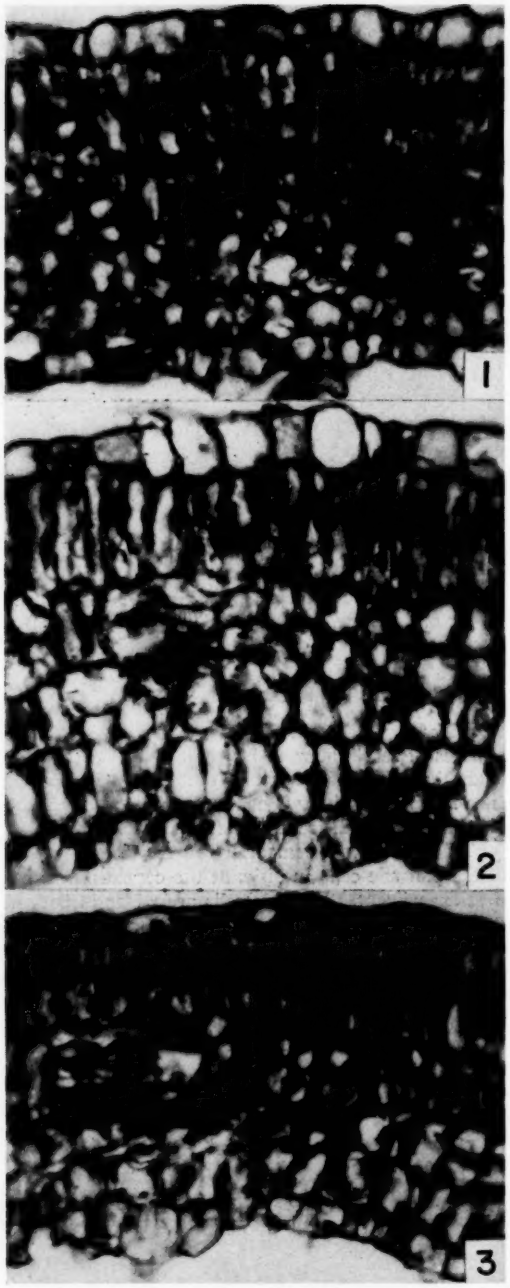
TABLE I

GROWTH OF DISKS AND WIDTH OF PALISADE CELLS FROM BEAN LEAVES

TREATMENT	GROWTH OF DISKS (MM)	WIDTH OF PALISADE CELL (μ)
None	1.42 ± 0.04*	6.53 ± 0.11*
2.0×10^{-4}M Kinetin	2.53 ± 0.05	8.94 ± 0.22
0.4×10^{-4}M Kinetin	2.22 ± 0.15	8.69 ± 0.28
12 Min red light	2.39 ± 0.08	6.94 ± 0.19

* Standard error

[1] Received August 7, 1959.



FIGS. 1-3. Cross section of disks from bean leaves 48 hours after placing in nutrient solution (480 X). Figure 1, control; figure 2, $2x10^{-4}$ M kinetin added to nutrient solution. Figure 3, 12 minutes red light given at time of placing in nutrient solution.

indicating that cells enlarged more in all directions. Neither of the treatments produced any observable changes other than size.

The second experiment also indicates that disk enlargement is increased by either kinetin or exposure to red light (table II). Growth promotion by kinetin was significant at the 5 % level in this experiment. Smaller disks were used in the second experiment than in the first. The darkroom was considered adequate at the time of the first experiment. However, since Miller (4) found that in the germination of lettuce seed the effectiveness of kinetin was increased by small amounts of light, some improvements were made in the darkroom before the second experiment. It may be that a decreased exposure to light in the second experiment reduced the kinetin effect. The disks grown in a solution of kinetin and exposed to red light were slightly larger (significant at the 5 % level) than disks receiving only one of the treatments.

Results in the second experiment were in agreement with those of the first in respect to cell enlargement (table II). Moreover, adding kinetin to the nutrient solution plus exposure to red light resulted in greater increase in cell width (significant at the 1 % level) than either treatment alone.

TABLE II

GROWTH OF DISKS AND WIDTH OF PALISADE CELLS FROM BEAN LEAVES

MIN RED LIGHT	M CONC KINETIN	DISK GROWTH (MM)	PALISADE CELL WIDTH (μ)
0	0	0.40 ± 0.07*	6.16 ± 0.20*
5	0	1.30 ± 0.08	6.44 ± 0.08
10	0	1.19 ± 0.12	5.80 ± 0.12
0	10^{-4}	0.88 ± 0.12	7.22 ± 0.12
5	10^{-4}	1.58 ± 0.16	8.62 ± 0.24

* Standard error

DISCUSSION

This study is in agreement with the previously reported finding that either kinetin treatment or exposure to red light produces increased expansion of disks of bean leaves. Miller (3) and Skoog and Miller (6) attributed the kinetin effect to greater cell enlargement, but published no data. Our results substantiate their explanation and demonstrate the extent of the increased cell enlargement. In addition, this study shows that cells from disks treated with red light were no larger than the control. The larger size of the disks after red light treatment must be due to an increase in the number of cell divisions.

This investigation was initiated to determine if kinetin and red light influenced growth through the same or a similar mechanism. If kinetin merely substituted for red light, the growth pattern resulting from either treatment would be the same. Our re-

sults show that kinetin stimulated growth by increased cell enlargement, but that red light stimulated growth by an increased rate of cell division. As stated earlier other investigations (4,5) have given some evidence that kinetin and red light are probably not involved in the same reaction. Our results further substantiate this concept and show conclusively that kinetin does not merely substitute for red light.

Although the types of growth resulting from kinetin and red light are different, an interaction between kinetin and red light may exist. Disks treated with both red light and kinetin were larger than if given either treatment alone. If kinetin stimulates greater cell enlargement and red light increases the rate of cell division, this result would be expected. With this simple relationship the cells should be the same size with both treatments or with kinetin alone. However, the cells from disks receiving both red light and kinetin were significantly larger ($P<0.01$) than those from disks treated with kinetin alone. These results indicate that there is an interaction between kinetin and red light. Further research is needed to determine the nature of such an interaction. The results presented here indicate that the answer may be found in the complicated relationship between cell enlargement and cell division.

Summary

I. Red light increased expansion of disks cut from etiolated bean leaves, but did not significantly alter the size of palisade cells.

II. Kinetin increased the expansion of disks cut from etiolated bean leaves and also caused a marked increase in cell size.

III. The action of kinetin and red light together caused a greater increase in size of disks cut from etiolated bean leaves than either treatment alone.

IV. Kinetin did not physiologically replace or substitute for red light.

V. An interaction between the effects of kinetin and red light probably exists, but its nature has not been determined.

Literature Cited

1. Duncan, D. B. 1955. Multiple range and multiple F. tests. Biometrics 11: 1–42.
2. Hendricks, S. B. and H. A. Borthwick 1955. Photoresponsive growth. In: Aspects of Synthesis and Order in Growth, D. Radrick, ed. Pp. 149–169. Princeton University Press, Princeton, N. J.
3. Miller, C. O. 1956. Similarity of some kinetin and red-light effects. Plant Physiol. 31: 318–319.
4. Miller, C. O. 1958. The relationship of kinetin and red-light promotion of lettuce seed germination. Plant Physiol. 33: 115–117.
5. Scott, R. A., Jr. and J. L. Liverman 1956. Promotion of leaf expansion by kinetin and benzylaminopurine. Plant Physiol. 31: 321–322.
6. Skoog, F. and C. O. Miller 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 9: 118–131.

AUTOMATIC DEVICE FOR CONTROLLING LENGTHS OF LIGHT AND DARK PERIODS IN CYCLES OF ANY DESIRED DURATION [1]

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Since the discovery of photoperiodism by Garner and Allard (2), scientists have been concerned with controlling the length of day to which experimental plants or animals are exposed. If plants or animals are grown in rooms with artificial illumination it is possible to control day length precisely by operating lamps on an ordinary time clock. The experiment becomes more complicated, however, if one wishes to expose a series of experimental organisms to different day lengths in order to compare the effects of different treatments. One may operate a series of small chambers each containing an experimental group and control illumination of each chamber by a time clock. This becomes expensive; problems of temperature control often become serious. Another procedure is to have an experimentally controlled illuminated room with an adjacent dark room so the organisms can be moved from the illuminated room to dark room and back again at desired intervals. This requires manual manipulation at specified times, twice a day for each treatment, and care must be exercised that the treatments are shifted at exactly the correct time each day.

Most of the literature on photoperiodism has dealt with the influence of duration of light and dark periods in cycles of 24 hours. Recently, however, considerable interest has developed in the effects of cycle lengths other than 24 hours duration (1, 3). Such experiments have presented extreme difficulties in manipulation. If the organisms are to be transferred manually from light to dark room or vice versa, it is necessary for someone to be available night and day throughout the experimental period. Failure to shift one group of organisms at the correct time may lead to failure of the entire experiment.

We have devised a simple apparatus for automatically shifting plants from light to darkness and vice versa with cycle lengths of any desired duration. While the apparatus was designed for work with plants, it should work equally well with most small animals. We have called this apparatus a photocycler.

MATERIALS AND METHODS

The basic unit of the photocycler is a four-drawer filing cabinet. This steel cabinet is 52 in high, 18 in wide, and 26.5 in deep. The upper two drawers and bottom drawer of the cabinet are discarded while the third drawer is left intact. The third drawer holds the plants. It is also used as a carrier drawer to move plants in and out of the cabinet body. A plywood facing is placed on the front side of the third drawer so that when the drawer is closed the chamber becomes a perfect darkroom. A separate cover is fixed over the opening of the fourth drawer to make the bottom side of the chamber light tight. When

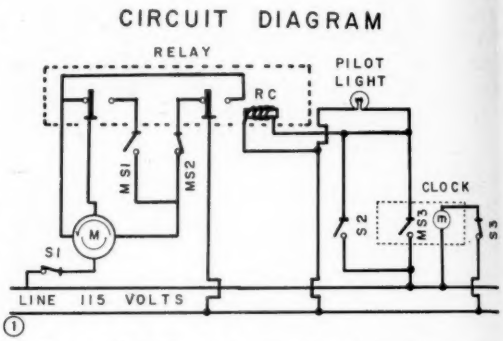

FIG. 1. CIRCUIT DIAGRAM. The wiring diagram in a unit of photocycler is shown in the figure. The circuit represents the operation when the relay is just de-energized and the drawer which was completely open, has started to close. When the drawer was completely open, the motor was stopped because the micro switch (MS1) was open. At this position the relay was energized and the current was being fed to the relay coil (RC) through the clock micro switch (MS3) which was closed. As soon as the clock micro switch (MS3) was opened by the clock motor (m), the relay was de-energized and the motor (M) was reversed. Consequently the drawer started closing. Before the drawer will be completely closed the motor (M) will stop because micro switch (MS2) will open. The two micro switches (MS1 and MS2) are normally closed. The drawer will remain closed as long as the relay remains de-energized. During the period when the relay was energized the pilot light was on. This light is also used to adjust the clock timings without disturbing the carrier drawer. After the clock is disconnected the drawer can be opened and closed manually through a switch (S2). A separate switch (S3) is provided to disconnect the clock motor after the experiment in a particular unit is over. The reversible motor can be stopped at any desired position by another switch (S1).

[1] Received August 14, 1959.

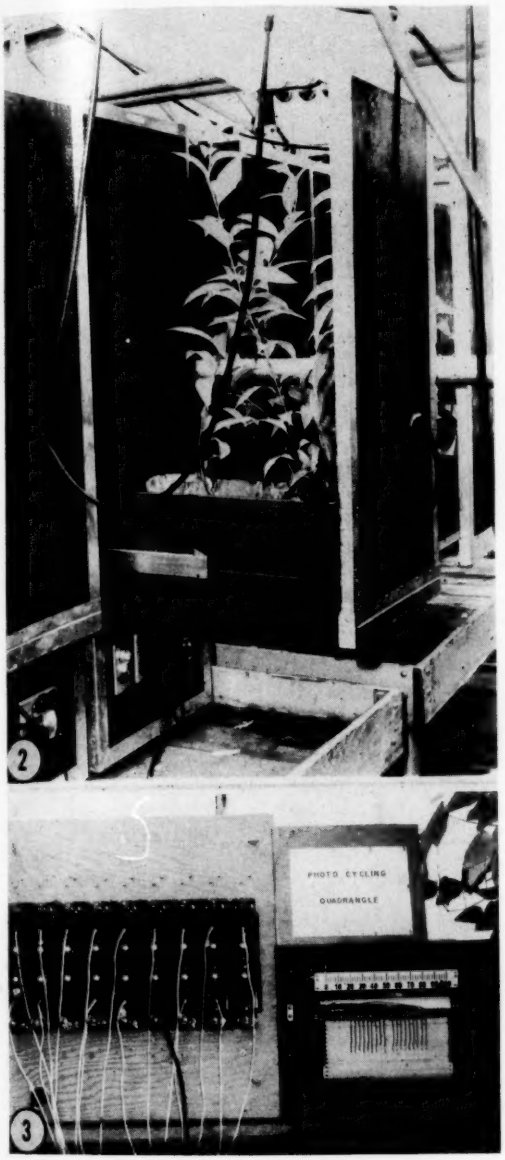

FIG. 2. PHOTOCYCLER. The picture was taken when all the 18 photocyclers were in actual operation. One unit shows the plants under illumination. The tubing through which plants are watered automatically is also seen. Beneath the drawer of each photocycler an attached time clock is shown.

FIG. 3. PAPER-TAPE TIMING MECHANISM. Potentiometer with paper tape mechanism (*right*), and control panel (*left*). Each of the 18 Photocyclers is connected to the potentiometer by a separate wire. Opening and closing of a particular unit is shown by the pilot light provided for each circuit separately on the control panel.

the carrier drawer is opened the plants are exposed to illumination from power-groove fluorescent lamps (fig 2).

The space provided by removal of the fourth drawer houses the necessary machinery. A rack is mounted on the bottom of the carrier drawer, which is moved by a pinion mounted on a reversible motor. The carrier drawer is opened and closed automatically by activating the reversible motor in one or the other direction through a magnetic relay. When the relay is energized the drawer moves out until stopped by a limit switch and remains in open position while the relay is not de-energized (see fig 1). As soon as this relay is de-energized the motor is reversed and the drawer is automatically closed. The current to the magnetic relay coil is controlled by an electric clock.

Two micro-switches are provided to limit the travel of the carrier drawer while moving in or out of the cabinet body. Before the drawer is fully open a knob attached to the bottom of the drawer hits a micro-switch which opens the circuit of the motor. The motor stops and the drawer remains in the open position. Similarly, before the drawer is completely closed the knob on the bottom of the drawer hits and opens another micro-switch which breaks the circuit of the motor. The drawer remains in closed position till the motor is again reversed (fig 1). To safeguard the motor from damage by overtravel of the carrier drawer, a clutch mechanism is provided on the pinion mounted on the motor.

An automatic watering system is provided. A steel tray is placed in the bottom of each drawer and the pots stand in it. Water flows into the tray through an inlet connected by a dark flexible tubing from outside of the cabinet. This flexible tubing allows free movement of the drawer and is connected to a main supply pipe. The valve on this pipe is controlled by a time clock which turns the water on for a predetermined period twice a day. The plants in the tray are watered by subirrigation. The excess of water drains out of the tray through an outlet to the outside of the cabinet. Plants in all the 18 photocyclers are watered automatically twice a day whether they are in light or darkness (fig 2).

Two timing methods have been used to activate the motor to close or open the drawer. One method involves the use of a time clock for each cabinet. Time clocks can be obtained which operate on almost any desired cycle length. Two discs on the face of the clock may be adjusted to turn a circuit to the relay coil on or off at any desired period during the cycle. With such time clocks a given cabinet may be set to operate indefinitely on a given cycle length with a desired light period and dark period in each cycle.

Another method provides more flexibility, but the control program for all cabinets must be set for each experiment. In this method a roll of paper tape which is used on an automatic recording potentiometer serves as a time chart for an entire set of cabinets. The relay circuit switch (fig 1 MS3) which controls the operation of a cabinet is activated by an impulse relay. The circuit of the impulse relay passes through

a wire in contact with the surface of the paper on the face of the recording device (fig 3). Underneath the paper is a metal plate and when the wire is in contact with the plate, the controlling switch is moved on or off and the cabinet drawer either opens or closes depending upon its position. By punching a series of holes in the paper tape, the opening and closing of the drawer may be controlled for any desired period of time in any given experiment.

An entire experiment involving 18 different cabinets has been set up on a single roll of paper. Experiments can be devised whereby the cycle lengths of a given cabinet may be varied from one cycle to the other. Short cycles may be alternated with long cycles. In fact, any conceivable regime of alternating light and darkness may be set up in this control mechanism provided, of course, the photoperiods and the dark periods are not too short. The opening and closing of the drawer takes approximately ten to fifteen seconds. Although a recording potentiometer is used in our apparatus to move the paper tape at a desired speed, separate units are available on which the paper tape may be used for setting the operation of the experiment. An independent clock unit using the paper tape mechanism can be easily furnished for an entire set of photocyclers.

Acknowledgements

The authors would like to thank Mr. Harvey W. Tomlin for helping in the design and construction of the apparatus. They would like to thank Dr. R. G. Lincoln for useful suggestions in designing the paper tape clock mechanism.

Literature Cited

1. Blaney, L. T. and K. C. Hamner 1957. Interrelations among effects of temperature, photoperiod, and dark period in floral initiation of Biloxi soybean. Bot. Gaz. 119: 10–24.
2. Garner, W. W. and H. A. Allard 1920. The effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. Jour. Agr. Research 18: 553–607.
3. Nanda, K. K. and K. C. Hamner 1958. Studies of the nature of the endogenous rhythm affecting photoperiodic response of Biloxi soybean. Bot. Gaz. 120: 14–25.

1960 MEETING OF SOUTHERN SECTION OF AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS. On Feb. 1 through 3 the Southern Section of the American Society of Plant Physiologists met with the Association of Southern Agricultural Workers at Birmingham, Ala. All sessions were well attended. The program included 27 contributed papers and two symposia.

A symposium on physiological genetics was led by Dr. Howard Teas, University of Florida. Papers were presented by Mr. Luther S. Bird and Dr. Howard E. Joham, Texas Agricultural Experiment Station; Dr. Charles Lewis, U.S.D.A., Beltsville, Md.; Mr. Theodore Holmsen and Dr. Howard Teas, University of Florida, and Mr. Ellsworth C. Maine and Dr. Arthur Kelman, North Carolina State College. These papers were recorded; copies will be available from the secretary-treasurer at the cost of $1.00. A second symposium was held jointly with the American Society of Agronomy. The topic of this session was the "Chemical Control of Plant Growth". Presiding were Dr. R. E. Blaser, Virginia Agricultural Experiment Station and Dr. Howard E. Joham, Texas Agricultural Experiment Station. Papers were given by Dr. Wayne C. Hall, Texas Agricultural Experiment Station; Dr. D. Harold McRae, Rohm-Haas Co.; Dr. L. F. Seatz, Tennessee Agricultural Experiment Station; and Dr. P. H. Harvey, North Carolina Agricultural Experiment Station.

On Wednesday morning the annual Plant Physiologists Breakfast was held. Copies of ANNUAL REVIEW OF PLANT PHYSIOLOGY, vol. 10, 1959, were presented in the first annual graduate student award. The award was given to the two graduate students presenting the best papers. Recipients of these awards were Mr. Theodore Holmsen, University of Florida, for the paper, "An Estimate of Protein Turnover in Tobacco", and Mr. Gene Guinn, Texas Agricultural Experiment Station, for the paper, "The Influence of Chelation on the Uptake and Distribution of Fe, Mn, Zn, and Cu in the Cotton Plant". The annual breakfast address, entitled "Botany in the English University", was given by Dr. Aubrey Naylor, Duke University.

Officers elected for 1960 were: Chairman, Dr. Robert E. Burns, U.S.D.A., Experiment, Ga.; Vice-Chairman, Dr. Howard E. Joham, Texas Agricultural Experiment Station; and Secretary-Treasurer, Dr. Robert D. Powell, University of Florida. Elected members of the Executive Committee are Dr. Howard Teas, University of Florida; Dr. Aubrey Naylor, Duke University; and Dr. Wayne C. Hall, Texas Agricultural Experiment Station. Dr. Teas is the Southern Section representative to the American Society of Plant Physiologists.—ROBERT D. POWELL, *Department of Botany, University of Florida, Gainesville, Fla.*

INTERNATIONAL GROWTH SYMPOSIUM AT PURDUE UNIVERSITY. An international symposium on growth will be held June 16, 17, and 18, at Purdue University, Lafayette, Ind. Some 33 speakers from six nations will discuss basic questions of molecule, cell, and organism growth.

For further information address: Dr. M. X. Zarrow, Department of Biological Sciences, Life Science Bldg., Purdue University, Lafayette, Ind.

AGRICULTURAL METEROLOGY CONFERENCE IN KANSAS CITY. On May 18 and 19 the Third Conference on Agricultural Meterology will be held in Kansas City, Mo. One session will be devoted to bioclimatic relationships with reference to ecology and phenology. Sessions on other phases of agricultural meterology also are planned.

For further information write to the office of the executive secretary, American Meterological Society, 45 Beacon St., Boston 8, Mass.

FIRST CANADIAN SOCIETY MEETING. The Canadian Society of Plant Physiologists will hold its first scientific meeting at the University of Toronto on June 2 and 3, 1960. The proceedings will include a symposium on developmental physiology. Address inquiries to the secretary-treasurer, Dr. D. F. Forward, Department of Botany, University of Toronto, Toronto, Canada.

SOCIETY FOR BIOLOGICAL RHYTHM. The Seventh International Conference of the Society for Biological Rhythm will be held in Siena, Italy, on September 5-7, 1960. The program will include invited and contributed papers in the fields of Botany, Zoology, and Medical Science. Information and application forms may be obtained from the secretary, Dr. Arne Sollberger, Department of Anatomy, Caroline Institute, Stockholm 60, Sweden.

American participants at the Congress may apply for travel grants from Dr. R. P. Forster, Program Director for Regulatory Biology, National Science Foundation, 1951 Constitution Avenue, Washington 25. D. C.

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Strive for specific and concise expression. Use active verbs. Avoid complicated sentence structure. Write in a straightforward manner. Use care in preparing the original manuscript to obviate subsequent delays in publication because of the need for manuscript revision.

B. Length Beyond ten pages (including tables, figures, and literature citations) authors will be billed $20 per page for overrun. Approximately four double spaced typewritten pages (pica type) equal one page of printed type.

C. Footnote References These should be avoided in the body of the text, but if a footnote must be used, it should be numbered with an Arabic numeral and typed at the bottom of the page on which reference to the footnote is made.

D. Acknowledgements Do not include acknowledgements in a footnote; they belong under their own heading following the SUMMARY and preceding LITERATURE CITED.

E. Punctuation

1. Periods and Commas These are the most often used and misused punctuation marks; the period is not used enough, the comma too often. Avoid complicated sentences which require many commas, colons, or semicolons. Break long sentences into brief, concise sentences which will be short and easily understood.

2. Quotation Marks Avoid the urge to use excessive quotation marks. Often a word is used in a sense slightly different from its ordinary meaning. This does not necessarily warrant the use of quotation marks. In case a term is used in an unconventional way and the ambiguity might lead to misunderstanding, use quotation marks only when the unfamiliar meaning is first explained.

3. Hyphens Use sparingly.

F. Subtopics Use this order and style to number subtopics:

I. I)
A. A)
1. 1)
a. a)

G. Scientific Names The complete botanical name (genus, species, and authority for the binomial) of all experimental plants should be included, preferably under MATERIALS AND METHODS. Refer to the most recent International Code of Botanical Nomenclature for rules governing the use of scientific names.

H. Abbreviations In place of certain unwieldy chemical names abbreviations may be used as a convenience. The writing, however, must remain readily comprehensible and unambigious. For the most part only widely used abbreviations should be employed (e.g.: ATP, RNA, TPN). Standard chemical symbols may be used without definition: Ca, NaOH. All others should be defined when they first appear. Example: The reaction was thought to involve flavin adenine dinucleotide (FAD). If your article uses several abbreviated forms it is permissible to define them all in a single footnote where the first abbreviation is introduced.

In no case should the number of nonstandard abbreviations be so large that the reader is forced to learn a special vocabulary in order to read your article easily. Abbreviations should not frustrate the reader; they should be used for his convenience, not the author's convenience.

Avoid abbreviations in title and summary. Because these parts frequently are translated into foreign languages, undefined abbreviations may be confusing.

I. Chemical Formulas It is expensive (sometimes impossible) to set formulas in type. Also there is danger of introducing errors. Submit structural formulas or involved reaction sequences as clearly executed India ink drawings which can be reproduced photographically. Indicate where these are to be inserted into the text. Alternatively a complex reaction diagram or flow sheet may be treated as a regular figure, given a number, and supplied with a legend.

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Carefully type copy in accordance with instructions. Give special attention to the form used by the Journal for tables, figure legends, and literature citations. Consult recent editions of PLANT PHYSIOLOGY for proper placement of main headings, paragraph headings, and subheadings, and for the use of abbreviations.

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e. Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 min, three to ten degrees. Exceptions: tables, figures, graphs.

F. FRACTIONS Write out and hyphenate nonprecise simple fractions: two-thirds, *but* half strength. Exceptions: tables, figures, graphs, legends, fractions in parentheses. Often it is desirable to use decimals instead of fractions. For values less than one, insert a zero before the decimal point: 0.7 g *not* .7 g.

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Check all citations (such as title, volume number, pages, and date) for completeness. Check foreign titles for diaresis and other markings. Make certain that all citations are referred to in the text.

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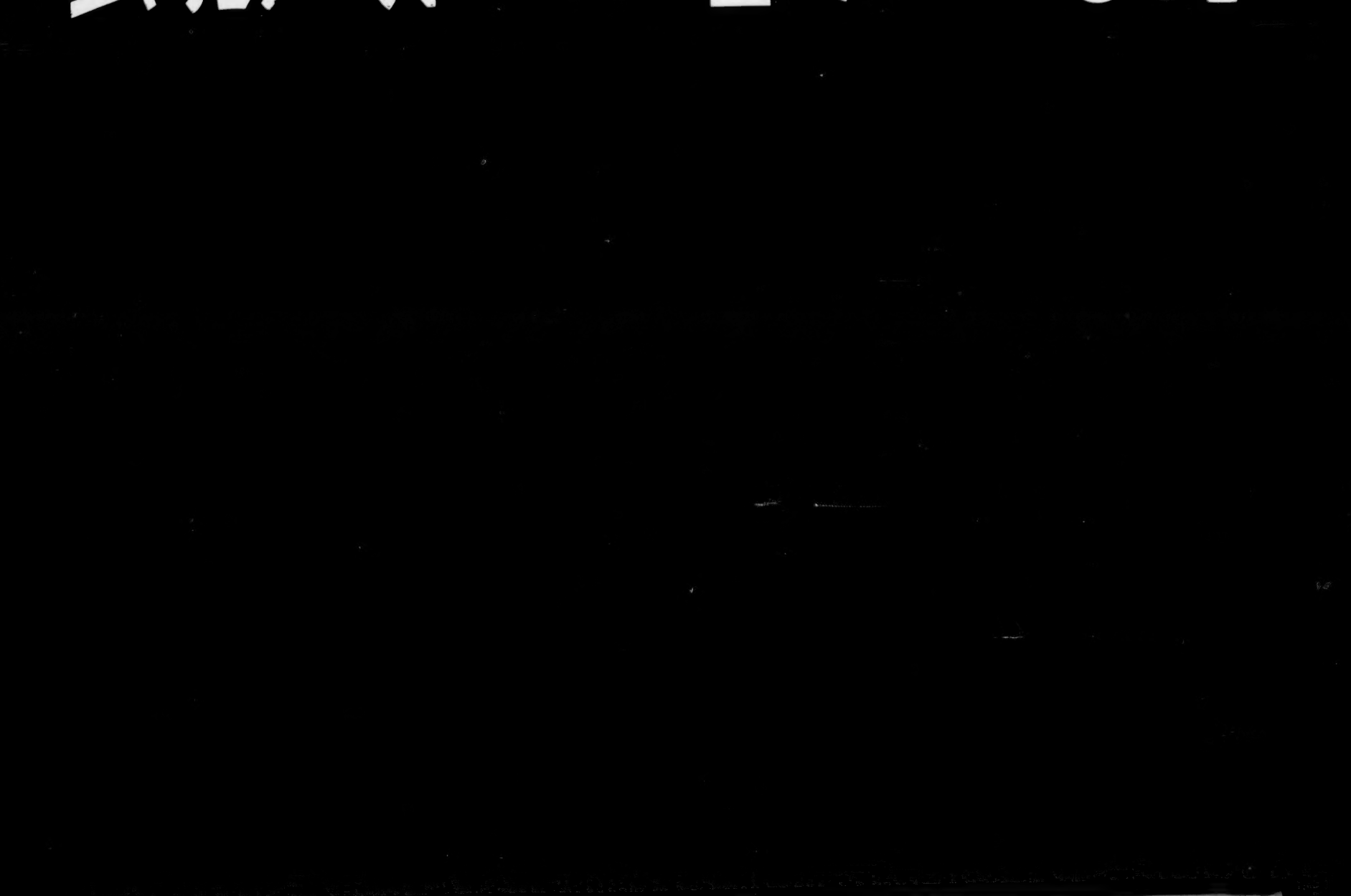
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